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CLINICAL AND EXPERIMENTAL

EXPERIMENTS ON THE MAINTENANCE OF MINERAL DENSITY IN THE SKELETON*

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IN A series of experiments previously recorded[9] we have investigated the efficacy of aqueous preparations of the antirachitic principle in maintaining or restoring a healthy structural appearance in the growing ends of the long bones of rats fed on a mineral deficiency diet. Despite the restoration, these aqueous preparations, like the oleaginous preparations of vitamin D, were incapable of maintaining adequate mineralization of compacta and spongiosa of the shafts in the absence of a sufficient mineral intake. Owing to the fact of growth there results a progressive osteoporosis roentgenographically and histologically apparent. Ash-values become relatively low and a generalized neuromuscular irritability supervenes already described by Templin and Steenbock.[8]

Having learned that aqueous preparations are both possible and efficacious we turned to the mechanism of calcium deposit in the growing ends of bones, utilizing for our study in vitro methods in order to secure simplicity of control and eliminate the complications inseparable from tissue growth in the living body.

TECHNIC OF EXPERIMENTS

White rats, bred in the laboratory, were weaned at one month of age and immediately placed upon the rachitogenic diet No. 2965 of Steenbock and Black.[6] After one month on this feeding the animals were killed. The upper ends of both tibiae were dissected out under sterile conditions, freed from adhering tissues and split longitudinally into halves. Thus each animal served for one

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experiment which utilized the four comparable bone fragments. Each fragment was immersed in sterile fluid of known volume and controlled pH for sixteen hours at a constant temperature of 38° C.[7]

One fragment, the negative control, was immersed in sterile distilled water. A second, the positive control, was immersed for sixteen hours in a calcifying medium of the following composition[5] diluted to give the appropriate percentage of calcium glycerophosphate as noted in the table:

Sodium chloride	0.6 gm. per 100 c.c.
Sodium bicarbonate	0.03 gm. per 100 c.c.
Potassium chloride	0.04 gm. per 100 c.c.
Magnesium sulphate ($MgSO_4 7H_2O$)	0.025 gm. per 100 c.c.
Calcium chloride	0.010 gm. per 100 c.c.
Tribasic sodium phosphate	0.082 gm. per 100 c.c.
Calcium glycerophosphate	2.0 gm. per 100 c.c.

Enough of the inorganic basal solution was made up at the beginning to serve for all the experiments. The bicarbonate was added last and before this addition the solution was sterilized. Next the pH was adjusted to between 7.4 and 7.6 by equilibration with carbon dioxide.[4] With the addition of glycerophosphate the hitherto inorganic basal solution now becomes the organic basal medium and is thus defined in later paragraphs.

The third and fourth fragments were immersed in the organic basal medium for the same length of time and under identical conditions of temperature and hydrogen-ion concentration but to the medium was added the preparation the effect of which was to be investigated. Allowance was made for dilution so that the salt concentration of the organic basal medium should be identical with that of the positive control.

After the sixteen-hour immersion period the fragments were washed with saline, fixed in 10 per cent formalin, dehydrated, embedded in paraffin and sectioned. The sections were transferred to slides, stained with von Kossa's silver nitrate (1.5 per cent $AgNO_3$) for two minutes, then exposed to bright light, and mounted in balsam.[1]

An aqueous extract of the antirachitic principle was prepared by diluting 10 c.c. of viosterol 10,000X, generously furnished by Mead Johnson and Co., with a small quantity of acetone, triturating the mixture with talc, slowly adding water with continued trituration and filtering the product through dry filter paper.

The resultant filtrate was a colorless, slightly opalescent fluid, odorless and tasteless but nevertheless a reliable source of vitamin D as shown by the biological assay already reported.[9]

This aqueous preparation of activated ergosterol which we term aquasterol was added, in these experiments, to the organic basal solution described above. Aqueous control preparations of olive and cod liver oils were made by a technique identical with that used in preparing aquasterol.

With exceptions noted in the table we utilized, for the immersion of bone fragments, a total fluid volume of 5 c.c., representing 0.05 gm. or such amount of

calcium glycerophosphate as is noted by percentage in the table. The concentration of the immersion-bath was regulated by diluting the calcifying medium with sterile distilled water. To study the effect of vitamin D our aquasterol preparation was used as the diluent in place of distilled water. The experiments consisted in varying the amount of aquasterol and the calcium concentration in the immersion-bath.

Assessments of the mineralization both in test material and controls were made upon histological sections prepared as already set forth.[9]

At first arbitrary ratings from 0 to 60 were devised on the basis of, but not identical with, Dyer's histological standards.[3] Rating No. 10 identifies the

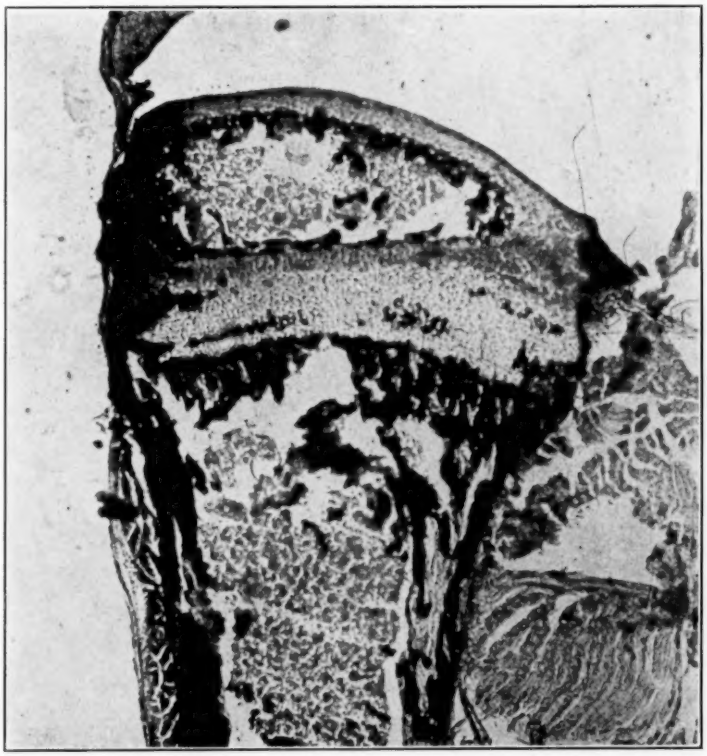

Fig. 1.—Experiment 1. Positive control. Tibia fragment No. 84. Organic basal (calcifying) medium without aquasterol. A practically unbroken cap of mineralization between spongiosa and hypertrophic zone of cartilage. Small irregular isolated areas of mineralization in hypertrophic zone itself.

average amount of restoration characteristic of immersion in the calcifying, non-aquasterol-containing medium of the positive control experiment. Rating No. 60 represents the calcification comparable with that of a normal bone, for aquasterol preparations never cause encroachment of mineralization into the diaphyso-epiphysial plane. Graded valuations were assigned to intermediate stages of mineralization. Further experience has shown that the method of evaluation, while important in the biological assay of vitamin D preparations, does not permit the fine discrimination necessary for this study. The method which has given us greatest satisfaction is a histological analysis. The growing

end of a long bone presents, on the shaft side of the diaphyso-epiphysial plane or zone 1, reserve cells of Dodds and Cameron,[2] a zone of proliferating cartilage formed by parallel columns of cells (zone 2, multiplying cells, Dodds and Cameron). Proximal to the zone of proliferating cartilage is the zone of hypertrophic cartilage, the cells of which appear swollen and their nuclei large and less deeply stained (zone 3, growing cells, Dodds and Cameron). The hypertrophic zone is normally sharply defined from the spongiosa.

This histological arrangement is equally apparent in healthy and in rachitic bones but, in the latter, the zones are wider, are often marred by degenerative

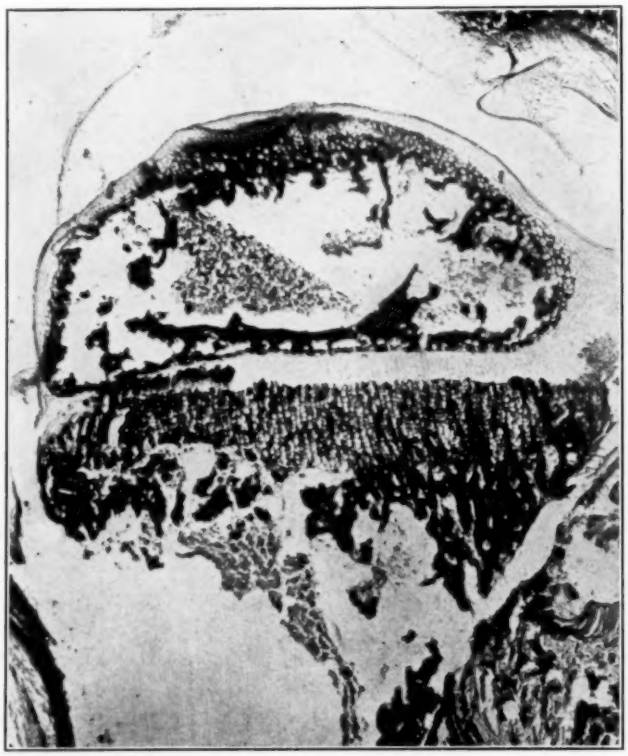

Fig. 2.—Tibia test fragment No. 85. Organic basal medium and aquasterol in equal parts. Uniform mineralization of hypertrophic zone. No mineralization of proliferative zone.

changes which may progress to actual cyst formation and to proliferative processes of osteoid tissue invading the demineralized spongiosa, the demarcation between which and the hypertrophic zone becomes irregular and ill-defined.

Most of our studies have been made on the proximal end of the tibia and our results are expressed in terms of the zones mineralized in that bone. We have included Figs. 1 to 3 as illustrations of the histological analysis upon which we have relied for the conclusions given in this paper.

EXPERIMENT NO. 1

Our first inquiry was the effect on mineralization of varying the amount of antirachitic principle in the immersion fluid. Experiment No. 1 (see Table),

utilizing fragments Nos. 83, 84, 85, 86, maintains in the calcifying bath a uniform concentration of calcium glycerophosphate (0.05 gm. in 5 c.c.) for all fragments except the negative control No. 83 which was immersed in distilled water. Fragment 84 was immersed in the organic basal solution diluted with water. For fragments 85 and 86 the diluent was aquasterol instead of distilled water but the amount of aquasterol used for No. 85 was two-thirds that used for No. 86.

In the negative control (No. 83) the mineralization of the fragment extends, however inadequately, through the spongiosa of shaft but does not penetrate the zone of hypertrophic cartilage. In the positive control (No. 84) the spongiosa

Fig. 3.—Tibia test fragment No. 86. Organic basal medium and aquasterol in proportions 1:3. Mineralization precisely as in Fig. 2 despite greater content of aquasterol.

may be more densely mineralized but the characteristic features are a practically unbroken cap of mineralization between spongiosa and the zone of hypertrophic cartilage, and secondly small irregular isolated areas of mineralization in the hypertrophic zone itself (Fig. 1).

In both test fragments (Nos. 85, 86) not only is there a denser mineralization of spongiosa but the zone of hypertrophic cartilage is mineralized uniformly throughout its extent and the unaffected zone of proliferative cartilage together with the diaphyso-epiphysial plane (zone of parallel rows of cartilage cells) remains as a narrow band, clear of mineral deposit. Moreover there is no appreciable difference in the degree of mineralization in these two fragments despite the difference in aquasterol concentration (Figs. 2 and 3).

This and similar experiments (2, 3, 5, 6, 7, 8, 10, 11, 12) demonstrate that, in the presence of an adequate amount of utilizable calcium in the calcifying medium, the addition of aquasterol intensifies the extent and density of mineralization. Increase of the aquasterol content of the calcifying medium beyond the effective point does not produce a corresponding improvement of the mineralization.

Cystic formations in the hypertrophic zone, particularly at the limit of the spongiosa, and osteoid penetration from the spongiosa into the hypertrophic zone are both frequently seen in rachitic bones. These pathological features have no real significance in our study. They merely indicate that the rachitic process was already more extensive than the stage required for in vitro studies.

EXPERIMENT NO. 2

This experiment was devised to investigate the effect of increasing the vitamin D and reducing the calcium concentration in the calcifying medium.

Fragment 87, the negative control, showed no calcification. The positive control, fragment 88, showed calcification of poor density widely distributed throughout the hypertrophic zone but not extending into the proliferative zone. Fragment 89 was immersed in a medium of calcium concentration identical with that used for fragment 88, namely 0.025 gm. per 5.0 c.c., but using 3.8 c.c. aquasterol instead of 3.8 c.c. of water as diluent. The calcification in this fragment was denser and more uniform in the hypertrophic zone than in fragment 88 and extended to the limit but not into the substance of the proliferative zone as in fragments 85, 86 though lacking the density of mineralization characteristic of these fragments.

The significance of this experiment is that even in the presence of a diminished calcium concentration the extent and density of calcification in the growth area are favorably influenced by the presence of vitamin D in the calcifying medium but the density of mineralization depends ultimately upon the calcium concentration of the medium.

EXPERIMENT NO. 3

Experiment No. 3 duplicates in all details experiment No. 1 with precisely the same results. For this experiment fragments 91, 92, 93, 94 were used.

EXPERIMENT NO. 4

In order to rule out any possible effect of oil itself on mineral deposition we substituted for aquasterol the filtrate of an aqueous trituration of olive oil.

Fragment 95, the negative control, presents a severe degree of rickets. Spongiosa and cortex are very poorly mineralized and the hypertrophic zone is not merely devoid of calcification but has undergone considerable destruction through the proliferation of osteoid tissue.

Fragment 96, immersed in a calcifying bath identical with that described for fragment 84 (i.e. 0.05 gm. calcium glycerophosphate in 5 c.c. of calcifying medium), shows a widely distributed calcification of poor density extending throughout the zone of hypertrophic cartilage. The osteoid tissue invading this zone is also lightly mineralized. Cortex and spongiosa are, however, no denser than in fragment 95.

Fragment 97 was immersed in the same medium as fragment 96 but the diluent used was the aqueous olive oil preparation instead of distilled water in the proportion one-half calcifying medium to one-half aqueous olive oil. The histological features of this preparation are identical with those of No. 96.

Fragment 98 was immersed in a similar medium of identical calcium concentration as fragments 96 and 97 but the amount of aqueous olive oil was increased to 3.8 c.c. in a total of 5.0 c.c. The calcium glycerophosphate content of the 5.0 c.c. was 0.05 gm. as for Nos. 96, 97. No calcification took place.

From these experiments therefore it is evident that olive oil, in itself, is without favorable effect on mineralization.

Aqueous triturations of cod liver oil prepared in the same manner show if anything a slightly greater mineralization than the corresponding positive control employing the organic basal solution alone (see Experiment No. 9).

EXPERIMENT NO. 5

This experiment, utilizing fragments 99, 100, 101, 102, was performed for determining the effect of minimal concentrations of aquasterol in the presence of a slightly increased calcium concentration.

Fragment 99 (negative control) shows no calcium deposit even in the hypertrophic zone. Fragment 100 (positive control), immersed in 5.0 c.c. of the calcifying medium containing 0.75 gm. of calcium glycerophosphate, shows a well mineralized hypertrophic zone up to but not into the proliferative zone. Fragment 101, immersed in a medium of nearly the same calcium concentration to which was added 1.00 c.c. of aquasterol (6.0 c.c. total volume equal to 0.075 gm. calcium glycerophosphate), shows much denser mineralization extending throughout the hypertrophic zone clear to the limit of the proliferative zone. Fragment 102, immersed in equal parts of the basal solution and aquasterol (5.0 c.c. containing 0.03 gm. calcium glycerophosphate), shows a mineralization equally extensive and of approximately equal density with fragment 101, though of definitely greater density than fragment 100.

Thus the presence of vitamin D, even in small amounts, enhances both the extent and density of mineralization but the presence of vitamin D in larger amounts will not substitute for a deficiency of calcium in the immersing medium.

That this is also evident in experiments on living animals was shown in a previous communication.[9]

EXPERIMENT NO. 6

In experiment No. 6 the aquasterol content was kept constant but the calcium concentration was diminished. For this experiment fragments 119, 120, 121, 122 were used. Fragment 122 was immersed in a medium of 4.0 c.c. volume containing 0.03 gm. of calcium glycerophosphate. This concentration is greater than that used for fragments 89 (experiment No. 2) and 102 (experiment No. 5) and gave practically identical results.

Fragment 121 was placed in a medium of 3.0 c.c. volume containing 0.015 gm. of calcium glycerophosphate. Mineralization is sparsely distributed throughout the hypertrophic zone and does not penetrate the zone of proliferative cartilage.

Fragment 120, immersed in a medium of 2.5 c.c. volume containing calcium glycerophosphate 0.0075 gm., shows very imperfect mineralization in the hypertrophic zone alone, the mineral being distributed erratically and in varying density along the boundary line between hypertrophic and proliferative zones.

Fragment 119 was used as a negative control.

In the presence of a constant amount of vitamin D, a variation in the calcium of the medium results in a corresponding difference in extent and density of mineralization.

The foregoing experiments illustrate three significant features of bone mineralization.

1. The in vitro effect of aqueous preparations of vitamin D in favorably affecting the mineralization cannot be explained by any physical action of inert oil.

2. In the presence of a constant amount of vitamin D, variation in total available calcium results in a corresponding difference in extent and density of mineralization.

3. In the presence of a constant total available calcium the increase of vitamin D beyond a certain definite level gives no corresponding increase in extent or density of mineralization.

To amplify and confirm the observations recorded in these experiments just set forth the following supplemental experiments were carried out.

Experiment 7 doubles the maximum total amount of calcium glycerophosphate utilized in experiment 6. The fragments used were Nos. 123, 124, 125. Fragment 125 shows a far heavier mineralization in the presence of aquasterol than fragment 124 which was immersed in the glycerophosphate solution diluted with water alone.

Experiment 8, utilizing fragments 140, 141, 142, supplements experiment 7 by doubling the amount of aquasterol, the calcium glycerophosphate in the medium being maintained at a level intermediate between those of experiments 6 and 7. The result is identical in principle with that of experiments 6 and 7.

Experiment 9 duplicates experiment 8 but utilizes an aqueous preparation of an official U.S.P. cod liver oil in place of aquasterol. The fragments employed were Nos. 155, 156, 157. No. 157 shows a heavier mineralization than the positive control No. 156. This result should be contrasted with that of the olive oil experiment No. 4.

Experiment 10 supplements experiment No. 2, using approximately one fourth the amount of aquasterol. Fragments 171, 172, 173 were employed. The results are identical in principle with those of experiment 2.

Experiment 11 duplicates experiment 6 but uses a slightly greater amount of aquasterol and a smaller amount of calcium. Fragments used were Nos. 175, 176, 177, 178. The results are identical in principle with those of experiment 6.

Experiment 12, using fragments 179, 180, 181, supplements experiments 7 and 8. This is intermediate in calcium content (i.e., volume of calcifying medium) between experiments 9 and 10 and is almost identical in calcium content with experiments 1 and 3. The results simply confirm those already stated.

TABLE I

OUTLINE OF DATA UPON THE TWELVE EXPERIMENTS RECORDED IN THIS PAPER

EXPERIMENT NO.	FRAGMENT NO.	TOTAL VOL. BATH C.C.	VOLUME CALCIFYING MEDIUM C.C.	VOLUME WATER C.C.	VOLUME EXP. SOLUTION C.C.	PERCENTAGE GLYCEROPHOSPHATE	RATING ON CALCIFICATION
1	83	5.0		5.0		0	0
	84	5.0	2.5	2.5		1.0	5
	85	5.0	2.5		2.5	1.0	50
	86	5.0	1.2		3.8	1.0	50
2	87	5.0		5.0		0	0
	88	5.0	1.2	3.8		0.5	20
	89	5.0	1.2		3.8	0.5	45
3	91	5.0		5.0		0	0
	92	5.0	2.5	2.5		1.0	5
	93	5.0	2.5		2.5	1.0	40
	94	5.0	1.2		3.8	1.0	40
4	95	5.0		5.0		0	0
	96	5.0	2.5	2.5		1.0	25
	97	5.0	2.5		2.5	1.0	25
	98	5.0	1.2		3.8	1.0	5
5	99	5.0		5.0		0	0
	100	5.0	5.0			1.5	40
	101	6.0	5.0		1.0	1.27	60
	102	5.0	2.5		2.5	1.5	60
6	119	2.0			2.0	0	0
	120	2.5	0.5		2.0	0.3	10
	121	3.0	1.0		2.0	0.5	25
	122	4.0	2.0		2.0	0.75	35
7	123	5.0		5.0		0	0
	124	5.0	4.0	1.0		1.2	40
	125	5.0	4.0		1.0	1.2	60
8	140	6.0		6.0		0	0
	141	6.0	4.0	2.0		1.0	30
	142	6.0	4.0		2.0	1.0	50
9	155	6.0		6.0		0	0
	156	6.0	4.0	2.0		1.0	30
	157	6.0	4.0		2.0	1.0	50
10	171	3.0		3.0		0	0
	172	3.0	2.0	1.0		1.0	30
	173	3.0	2.0		1.0	1.0	50
11	175	3.0		3.0		0	0
	176	3.0	0.5	2.5		0.25	15
	177	3.0	0.5		2.5	0.25	25
	178	3.0	0.25		2.75	0.125	5
12	179	5.0		5.0		0	0
	180	5.0	3.0	2.0		0.9	40
	181	5.0	3.0		2.0	0.9	50

In all experiments except Nos. 4 and 9 the experimental solution is aquasterol prepared in the manner previously described.[9]

In Experiment 4 the experimental solution is olive oil; in No. 9 it is a standard U. S. P. cod liver oil.

SUMMARY

1. Experiments 1, 4 and 9 recorded in the body of this paper illustrate the following points.

Aqueous preparations of vitamin D favorably influence the extent and density of mineralization in rachitic bone fragments immersed in a solution of organic calcium.

This effect cannot be attributed to surface tension or other physical properties of oil present in our aqueous preparations of vitamin D.

2. The remaining experiments demonstrate two further principles.

In the presence of a constant amount of vitamin D, variation of the organic calcium concentration results in a corresponding effect on extent and density of mineralization in the growing area.

In the presence of a constant organic calcium concentration in the calcifying medium a variation of vitamin D content does not produce corresponding effect on mineralization of the growing area.

3. In surveying the results of these experiments it is essential to keep in mind the fact that the part of bone under observation is the growing area only. The epiphysis proper and the shaft are not studied in this communication. Results evident in the growing area must not be presumed to hold for shaft or epiphysis. For these areas further specific studies must be designed.

REFERENCES

1. Carlton, H. M., and Haynes, F.: Histological Technique, London, Oxford Univ. Press, p. 164, 1926.
2. Dodds, G. S., and Cameron, H. C.: Structural Modifications of the Epiphyseal Cartilages in the Tibia and Other Bones, Am. J. Anat. **55**: 135, 1934.
3. Dyer, F. J.: The Standardization of Vitamin D by the Line Test, Quart. J. Pharm. & Pharmacol. **4**: 503, 1931.
4. Kramer, B., Shelling, D. H., and Orent, E. R.: Studies Upon Calcification in Vitro: I. Effect of Reaction on Calcification, Proc. Soc. Exper. Biol. & Med. **24**: 240, 1926.
5. Robison, R., and Soames, K. M.: The Possible Significance of Hexosephosphoric Esters in Ossification, VIII. Calcification in Vitro, Biochem. J. **24**: 1922 (supp. 1923), 1930.
6. Steenbock, H., and Black, A.: Fat Soluble Vitamins. XXIII. The Induction of Growth Promoting and Calcifying Properties in Fats and Their Unsaponifiable Constituents by Exposure to Light, J. Biol. Chem. **64**: 263 (see p. 274), 1925.
7. Shipley, P. G., Kramer, B., and Howland, J.: Studies Upon Calcification *in vitro*, Biochem. J. **20**: 379, 1926.
8. Templin, V. M., and Steenbock, H.: Vitamin D and the Conservation of Calcium, J. Biol. Chem. **100**: 209, 1933.
9. Venar, Y., and Todd, T. W.: The Efficacy of Vitamin D Administration in Aqueous Preparation, J. Nutrition **8**: 553, 1934.

GRANULOCYTOPOIETIC FRACTIONS OF YELLOW BONE MARROW*

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IN RECENT years the condition known as granulocytopenia has attained a prominent position in the medical world. While the etiology of the disease still remains obscure, the many agents employed for its treatment have met with varying degrees of success.[1-3] Of these therapeutic agents, yellow bone marrow has attracted much attention.

Watkins[4] has obtained encouraging results by oral administration of bone marrow at the onset of the disease. He gave daily doses of approximately 60 to 120 gm. of fresh yellow bone marrow, obtained from beef, until the blood assumed a normal picture, and smaller doses for several months later. Since large amounts of marrow were necessary to obtain a change in the leucocytic picture, and since in certain cases the patient was unable to digest and assimilate such large quantities, the use of the fresh marrow for this purpose was considered undesirable. Accordingly, a study was undertaken to separate the granulocytopoietic fraction from yellow bone marrow, and to determine its activity on experimental animals.

EXPERIMENTAL

Source of Bone Marrow.—The bone marrow used in these studies was obtained from fresh long bones of cattle. It was freed of blood, bony and fibrous material and then ground.

Preparation 1 (P-1).—Two kilograms of bone marrow were saponified with 6 liters of 3 per cent alcoholic potassium hydroxide at room temperature for twenty-four hours. The saponification was aided by mixing the solution with a mechanical stirrer. At the end of twenty-four hours the solution was filtered through a large fluted filter. The residue consisted chiefly of insoluble bony and fibrous tissues. An equal volume of distilled water was added to the filtrate which separated into two layers. The upper layer containing the nonsaponifiable fraction was saved and the lower soapy layer discarded. In order to free the concentrate from alkali, it was washed 5 times with 50 c.c. of distilled water and 5 times with 50 c.c. of 40 per cent alcohol. From the original 2 kg. of bone marrow, 100 gm. of the concentrate were obtained.

The product thus obtained was a yellow, oily liquid, being nearly odorless and having a slightly oily after-taste. It had a neutral reaction to phenolphthalein, gave a negative test for sterols, and had a specific gravity of 0.8626.

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It was soluble in 95 per cent alcohol, ether, petroleum ether chloroform, and only slightly soluble in methyl alcohol. This concentrate, when administered orally to animals having the granulocytopenic condition, showed marked granulocytopoietic activity.

The results are shown in Table I.

TABLE I

RESULTS ON P-1

RABBITS	DATES	CONDITION	SUBSTANCE ADMINISTERED	TOTAL LEUCOCYTES	POLYNUCLEAR NEUTROPHILES	POLYNUCLEAR NEUTROPHILES, DEGENERATED	MONOCYTES	TRANSITIONAL CELLS	BASOPHILES	EOSINOPHILES	SMALL LYMPHOCYTES	LARGE LYMPHOCYTES
1	4/ 9/35	*N	–	8,700	52	–	2	2	1	–	38	5
	4/ 9/35	O	–	–	–	–	–	–	–	–	–	–
	4/10/35	PO	–	5,100	28	2	2	–	–	–	60	8
	4/11/35	PO	5 c.c. P-1	2,900	20	–	3	1	–	–	67	9
	4/12/35	PO	–	8,900	50	–	4	2	1	–	37	6
	4/13/35	PO	5 c.c. P-1	9,150	8	2	1	–	1	–	80	8
	4/15/35	PO	5 c.c. P-1	8,500	45	–	2	–	–	1	45	7
	4/16/35	PO	5 c.c. P-1	9,700	51	–	3	1	2	–	33	10
	4/17/35	PO D	5 c.c. P-1	9,000	50	–	4	–	–	–	40	6
2	4/ 9/35	N	–	8,750	47	–	6	1	1	–	32	13
	4/ 9/35	O	–	–	–	–	–	–	–	–	–	–
	4/10/35	PO	–	7,200	16	–	–	1	1	–	71	11
	4/11/35	PO	5 c.c. P-1	8,750	16	–	3	–	1	1	70	9
	4/12/35	PO	5 c.c. P-1	15,500	57	–	–	3	–	–	28	12
	4/13/35	PO	5 c.c. P-1	9,050	48	–	4	–	1	–	39	8
	4/15/35	PO D	5 c.c. P-1	9,000	50	–	3	2	2	1	36	6
3	4/ 9/35	*N	–	8,700	46	–	2	1	–	1	40	10
	4/10/35	O	–	–	–	–	–	–	–	–	–	–
	4/12/35	PO	–	10,000	30	8	1	–	1	–	45	15
	4/13/35	PO	–	9,050	33	3	4	–	–	–	53	7
	4/13/35	PO	5 c.c. P-1	5,100	7	–	1	–	–	–	82	10
	4/15/35	PO	5 c.c. P-1	8,900	40	5	–	3	1	1	41	9
	4/16/35	PO	5 c.c. P-1	7,500	51	–	3	1	–	1	34	10
	4/17/35	PO	5 c.c. P-1	9,000	56	–	1	2	1	–	27	13
	4/18/35	PO D	5 c.c. P-1	9,300	49	–	5	–	1	1	40	4
4	5/ 6/35	N	–	9,350	45	–	1	3	2	–	46	3
	5/ 7/35	N	–	9,950	50	–	3	1	1	–	42	3
	5/ 8/35	O	–	–	–	–	–	–	–	–	–	–
	5/ 9/35	PO	– –	4,050	17	9	1	–	–	–	65	8
	5/10/35	PO	10 c.c. P-1	2,500	4	10	2	3	–	–	70	11
	5/10/35 P.M.	PO	–	6,000	40	2	4	1	1	–	45	7
	5/11/35	PO	10 c.c. P-1	4,550	19	–	1	3	2	–	59	16
	5/13/35	PO	10 c.c. P-1	8,500	47	–	2	–	–	–	41	10
	5/14/35	PO	10 c.c. P-1	9,000	52	–	1	1	1	–	40	5
	5/15/35	PO	10 c.c. P-1	7,050	48	–	3	–	–	1	38	10
	5/17/35	PO D	10 c.c. P-1	8,600	50	–	6	–	–	1	37	6

*N, normal; O, operated; PO, postoperative; D, discontinued.

Preparation 2 (P-2).—Two kilograms of bone marrow were saponified exactly as described under Preparation 1. One liter of distilled water was added

TABLE II

RESULTS ON P-2

RABBITS	DATES	CONDITION	SUBSTANCE ADMINISTERED	TOTAL LEUCOCYTES	POLYNUCLEAR NEUTROPHILES	POLYNUCLEAR NEUTROPHILES, DEGENERATED	MONOCYTES	TRANSITIONAL CELLS	BASOPHILES	EOSINOPHILES	SMALL LYMPHOCYTES	LARGE LYMPHOCYTES
5	8/ 6/35	*N	–	7,200	49	–	4	1	1	1	31	13
	8/ 7/35	O	–	–	–	–	–	–	–	–	–	–
	8/ 8/35	PO	0.1 c.c. P-2	31,000	1	23	5	1	–	–	67	3
	8/ 8/35	PO	–	10,800	–	–	–	–	–	–	–	–
	8/ 9/35	PO	0.1 c.c. P-2	13,830	41	8	6	1	2	–	39	3
	8/12/35	PO	0.1 c.c. P-2	30,950	28	1	1	2	1	–	66	1
	8/13/35	PO	0.1 c.c. P-2	8,900	52	–	2	1	2	–	36	7
	8/14/35	PO	0.1 c.c. P-2	8,000	48	–	3	–	–	–	37	12
	8/15/35	PO	0.1 c.c. P-2	9,500	50	–	2	2	1	–	35	10
	8/16/35	PO	–			–	–	–	–	1	42	4
	8/19/35	PO D	–	9,000	49	–	3	1	1	–	35	11
6	8/26/35	N	–	7,500	43	–	5	1	1	–	40	10
	8/26/35 P.M.	O	–	–	–	–	–	–	–	–	–	–
	8/27/35	PO	–	13,400	43	5	1	–	–	1	48	2
	8/28/35	PO	0.5 c.c. P-2	3,600	4	8	2	–	3	–	71	12
	8/29/35	PO	0.5 c.c. P-2	17,550	42	–	2	–	4	–	42	10
	8/30/35	PO	0.5 c.c. P-2	7,700	54	–	5	3	5	–	28	5
	9/ 3/35	PO D	0.5 c.c. P-2	8,000	53	–	3	1	1	–	34	8
7	10/ 2/35	N	–	9,400	52	–	2	1	–	1	37	7
	10/ 2/35 P.M.	O	–	–	–	–	–	–	–	–	–	–
	10/ 3/35	PO	–	6,800	12	59	5	1	–	–	16	7
	10/ 4/35	PO	0.1 c.c. P-2	4,000	–	25	3	–	–	–	67	5
	10/ 5/35	PO	0.1 c.c. P-2	9,350	48	24	–	–	–	1	22	5
	10/ 6/35	PO	0.1 c.c. P-2	10,750	54	4	7	2	2	–	25	6
	10/ 7/35	PO	0.1 c.c. P-2	9,000	51	–	5	3	1	1	30	9
	10/ 8/35	PO	–	7,500	48	–	3	1	–	–	38	10
	10/ 9/35	D										
8	10/ 2/35	*N	–	6,800	48	–	4	1	1	1	33	12
	10/ 2/35	O	–	–	–	–	–	–	–	–	–	–
	10/ 3/35	PO	–	6,500	7	58	–	–	3	–	32	–
	10/ 3/35	PO	–	6,150	1	23	4	1	1	–	66	4
	10/ 4/35	PO	0.1 c.c. P-2	3,900	4	12	–	–	–	–	80	4
	10/ 4/35 P.M.	PO	0.1 c.c. P-2	6,800	37	2	3	3	1	2	48	4
	10/ 5/35	PO	0.1 c.c. P-2	6,350	52	–	3	1	3	1	35	5
	10/ 6/35	PO	0.1 c.c. P-2	7,200	43	–	–	–	2	–	47	8
	10/ 7/35	PO	–	8,500	50	–	1	1	1	1	37	9
	10/ 8/35	D										
9	10/ 2/35	N	–	8,900	53	–	2	3	–	1	34	7
	10/ 2/35	O	–	–	–	–	–	–	–	–	–	–
	10/ 3/35	PO	–	7,700	21	12	2	–	1	–	50	14
	10/ 4/35	PO	–	21,900	6	29	3	–	1	–	57	4
	10/ 4/35	PO	0.1 c.c. P-2	14,250	4	6	–	–	3	–	80	7
	10/ 5/35	PO	0.1 c.c. P-2	9,500	15	5	2	2	1	1	70	4
	10/ 6/35	PO	0.1 c.c. P-2	8,150	20	5	4	1	–	–	64	6
	10/ 7/35	PO	0.1 c.c. P-2	8,000	48	1	3	–	2	–	36	10
	10/ 8/35	PO	–	7,200	46	–	2	4	–	–	43	5
	10/ 9/35	PO	–	9,100	49	–	–	1	2	1	36	11

*N, normal; O, operated; PO, postoperative; D, discontinued.

to the saponifying solution, and after shaking thoroughly, 1 liter portions were extracted with 500 c.c. of petroleum ether (boiling point 40° to 50° C.). After the ether layer separated out, the underlying soap solution was drawn off. When all the saponifying solution was thus extracted, the petroleum ether solution was resaponified with 3 liters of 3 per cent alcoholic potassium hydroxide at room temperature for twenty-four hours. The soap formed by this procedure was insoluble in the liquid medium and was easily separated by filtration aided by suction. The soap was washed 3 times with 50 c.c. portions of petroleum ether. The addition of 1 liter of distilled water to the filtrate caused the formation of 2 layers. The upper layer was saved and the lower discarded. One liter portions of the petroleum ether solution were washed 5 times with 100 c.c. of 40 per cent alcohol; when all of the ether solution was thus washed, it was collected and distilled off under diminished pressure at 30° C. The resulting product had a slight cloudiness, but after filtering through a layer of asbestos it remained clear. Twenty grams of the concentrate were obtained from the original 2 kg. of bone marrow.

This concentrate was a brilliant amber, oily liquid, being odorless and imparting a faint oily taste. The specific gravity was 0.8276. It gave a neutral reaction to phenolphthalein, a negative test for sterols, and it was soluble in 95 per cent alcohol, ether, petroleum ether and chloroform. When this substance was administered intramuscularly to animals which showed a condition of granulocytopenia, a marked granulocytopoietic activity was observed within four hours.

The results are given in Table II.

Etiology of Granulocytopenia.—Since the disease granulocytopenia was first described in Germany in 1922[5] and in the United States in 1924,[6] many efforts[6-20] have been made to determine its cause. Many theories of the etiology of this disease have been advanced, namely, dietary deficiency,[7, 8] hormonic dysfunction,[9-12] use of certain coal-tar derivatives,[13-17] and bacterial infection.[18-20]

METHODS OF PRODUCING GRANULOCYPTOPENIC CONDITIONS EXPERIMENTALLY

1. Chemical Method.—Granulocytopenic conditions could not be produced in rabbits and rats by the administration of hydroquinone, amidopyrine, or benzene.

2. Bacterial Method.—The procedure used was a modification of the method described by Dennis.[18] Ten cubic centimeters of a twenty-four-hour culture of *Staphylococcus aureus* were placed into a Naturalamb skin sac which had been moistened with distilled water. The open end of the sac was sealed by tying with a linen thread. The outer surface of the sac was sterilized by immersing several times in 70 per cent alcohol.

The normal total and differential leucocytic counts were determined on each animal. The rabbits were starved for twenty hours before the operation. A light ether anesthesia was administered, and the peritoneal cavity was exposed by incision, care being exercised to maintain aseptic conditions. A sac, prepared as described above, was placed into the lower part of the peritoneal cavity. The peritoneum and abdominal wall were sutured with plain catgut. Metal clips were used on the skin. A bandage was applied and held in position by a

binder. Blood for the counts was obtained from the marginal vein of the ear, and the smears were stained by Wright's method.

The granulocytopoietic effect produced by this procedure was determined by making total and differential leucocytic counts. The results of these counts were used as the criterion of the effects produced on the granulocytopoietic system by the bone marrow concentrates.

Forty-six rabbits were prepared according to this method. Of this number 17 developed a condition of granulocytopenia, 9 died without showing any change in the leucocytic picture, and 20 recovered without any apparent ill effects.

Rabbits 1, 2, 3 and 4.—Rabbits 1 and 2 (males) and 3 and 4 (females) each weighing about 2.5 kg. were prepared according to the method described. All developed the granulocytopenic condition and recovered upon daily oral administration of P-1.

The data are given in Table I.

Note: In these Tables the cells termed as polynuclear neutrophiles, degenerated, were polynuclear neutrophiles characterized by disintegration of the cell membrane and cytoplasm (presence of vacuoles) and a distortion of the nucleus. These cells stained very poorly.

Rabbits 5, 6, 7, 8, and 9.—Rabbits 5, 6 and 7 (females) and 8 and 9 (males) each weighing approximately 2.8 kg. were prepared as described. Rabbits 5 and 9 did not develop a typical condition of granulocytopenia. The second day after the operation Rabbit 5 had a total leucocytic count of 31,000 and Rabbit 9, 21,900. In both cases only a few normal polynuclear neutrophiles could be found. The animals were given 0.1 c.c. of P-2 intramuscularly daily for six days. At the end of this period, the blood picture of each animal returned to normal, and they showed apparent recovery. Two days after the operation Rabbits 6, 7, and 8 developed the granulocytopenic condition. Rabbit 6 was given 0.5 c.c. and Rabbits 7 and 8, 0.1 c.c. of P-2 intramuscularly daily for four days. At the end of the four-day period, the animals showed a normal blood picture and appeared to be in good health.

The data are shown in Table II.

Observations on the Absorption and Local Reaction of P-2.—Twelve full-grown rats and 3 rabbits were used. The right leg of each animal was shaved and the skin was cleansed thoroughly with soap and water. Under aseptic conditions 0.2 c.c. of P-2 was injected into the muscle of the leg daily for four days. Each injection was made at the same site.

The injections did not cause any local reaction as manifested by the absence of redness, swelling, and restlessness in the animals. Four days after the last injections were made the animals were killed, and upon autopsy the muscles into which the preparation was injected showed no gross abnormality. This indicates definitely that this substance is easily absorbed when injected into an animal intramuscularly.

DISCUSSION

From these studies, it is evident that by saponifying yellow bone marrow with alcoholic potassium hydroxide at room temperature it is possible to isolate

fractions which appear to possess granulocytopoietic properties. As described under Preparations 1 and 2, the active fractions were isolated by two methods, namely, by separation with distilled water and by petroleum ether extraction. The former method affords a comparatively simple means of separation. However, by the latter method it is possible to obtain the fraction in a more concentrated form. In addition, the foregoing data show that Preparation 1 is active when administered orally, and that Preparation 2 is active when administered intramuscularly to rabbits in which a condition of granulocytopenia was produced experimentally. Although a very highly active concentrate was prepared, it will be necessary to conduct further studies on the purification and concentration, before any suggestion of chemical composition can be made.

In this work, attempts to produce granulocytopenic conditions in rabbits and rats by the use of hydroquinone, amidopyrine, or benzene were unsuccessful. Consequently, a modification of the method employed by Dennis[18] was used and proved to be satisfactory. Although the condition obtained by this procedure may not be identical with that in human beings, it affords a convenient laboratory method of demonstrating the granulocytopoietic activity of yellow bone marrow concentrates.

CONCLUSION

Highly concentrated fractions, possessing granulocytopoietic activity, were prepared from yellow bone marrow.

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REFERENCES

1. Minter, M. M.: The Treatment of Agranulocytic Angina, Southwest Texas Med. **2**: 8, 1935.
2. Harris, W. H., and Schattenberg, H. J.: So-Called Agranulocytic Angina With Special Consideration of the Causal Agent, New Orleans M. & S. J. **88**: 288, 1935.
3. Burcky, F. W.: Agranulocytosis, Illinois M. J. **67**: 59, 1935.
4. Watkins, C. W.: Personal communication to the author in November, 1934.
5. Schultz, W.: Ueber eigenartige Halserkrankungen, Deutsche med. Wchnschr. **48**: 1494, 1922.
6. Lovett, B. R.: Agranulocytic Angina, J. A. M. A. **83**: 1498, 1924.
7. Langston, W. C., and Day, P.: Cited by Kracke, R. R., and Parker, F. P.: The Relationship of Drug Therapy to Agranulocytosis, J. A. M. A. **105**: 900, 1935.
8. Miller, D. D., and Rhoads, C. P.: Experimental Production in Dogs of Acute Stomatitis, Associated With Leukopenia and a Maturation Defect of the Myeloid Elements of the Bone Marrow, J. Exper. Med. **61**: 173, 1935.
9. Britton, S. W., and Corey, E. L.: Blood Cellular Changes in Adrenal Insufficiency and the Effects of Cortico-Adrenal Extract, Am. J. Physiol. **102**: 699, 1932.
10. Thompson, W. P.: Observations on the Possible Relation Between Agranulocytosis and Menstruation, With Further Studies on a Case of Cyclic Neutropenia, New England J. Med. **201**: 176, 1934.
11. Jackson, H., Jr., Merrill, D., and Duane, M.: Agranulocytic Angina Associated With Menstrual Cycle, New England J. Med. **210**: 176, 1934.
12. Rutledge, B. H., Hansen-Pruss, O. C., and Thayer, W. S.: Recurrent Agranulocytosis, Bull. Johns Hopkins Hosp. **46**: 369, 1930.
13. Weiskotten, H. G.: The Action of Benzol: The Normal Life Span of Neutrophile (Amphophile) Leukocyte (Rabbit), Am. J. Path. **6**: 183, 1930.
14. Kracke, R. R.: The Experimental Production of Agranulocytosis, Am. J. Clin. Path. **2**: 11, 1932.
15. Madison, F. W., and Squier, T. L.: The Etiology of Primary Granulocytopenia, J. A. M. A. **102**: 755, 1934.

16. Hoffman, A. M., Butt, E. M., and Hickey, H. G.: Neutropenia Following Amidopyrine: Preliminary Report, J. A. M. A. **102**: 1213, 1934.
17. Special Reports of the Council on Pharmacy and Chemistry, A. M. A.: The Relation of Amidopyrine and the Barbituric Acid Derivatives to Granulocytopenia, J. A. M. A. **102**: 2183, 1934.
18. Dennis, E. W.: Experimental Granulopenia Due to Bacterial Toxins Elaborated In Vivo, J. Exper. Med. **57**: 993, 1933.
19. Felson, J.: Bacillary Dysentery—Acute Fulminating Type With Marked Toxic Neutropenia, New York State J. Med. **35**: 1037, 1935.
20. Sholl, C. P.: Personal communication to the author in March, 1935.

A COMPARISON OF THE DIRECT VERSUS THE INDIRECT METHOD OF ESTIMATING THE LIPID COMPOSITION OF THE RED BLOOD CELLS*

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THE rôle of the red blood cells in lipid metabolism is a difficult one to evaluate. On the one hand there is evidence that the erythrocytes may play a prominent part, but evidence of a contradictory nature suggests that they are inert. The point of present practical importance is that the lipid content of the red blood cells is not the same as that of plasma or serum and that when changes occur in plasma or serum these same changes do not occur in the red blood cells. As a result the lipid analysis of whole blood alone may fail to reveal any marked abnormality when actually opposite changes of significance have taken place in the cells and plasma. In any complete investigation both the cells and plasma should be examined; if only one value is determined then plasma or serum is to be preferred to whole blood.

In a number of conditions the lipids of the red cells have been found to remain unaltered in spite of marked changes in plasma and on the basis of this information the cells have been considered to be inert in fat metabolism. This has been shown in the persistent lipemias of diabetes, pregnancy, and chronic hemorrhage.[3] Wendt[12] reported that a fat meal caused an increase in plasma lipids but not those of the erythrocytes. Boyd[8] and Boyd and Fellows[11] have presented data which demonstrates that although the lipid composition of plasma differs markedly between man, rabbits, and guinea pigs, yet the lipid composition of the red blood cells is the same in all three species.

In other investigations the red cells have been found to take a prominent part in lipid metabolism, and this information is sufficient to warrant their inclusion in any serious, complete study of the blood lipids. In the first place, Bloor[1] has shown that a copious meal of fat produces an increase in the cellular lipids. Boyd and Tweddell[10] found that the ordinary meals do not appreciably affect the lipids of the red cells but that certain curious changes occurred in the early morning hours between midnight and rising. In fever[6] the concentration of lipids in the erythrocytes varies oppositely to that of plasma,

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suggesting the red cells exert a sort of buffer action. Ether anesthesia[7] affects chiefly the red cells. Boyd and Wilson[9] have shown that lipids are carried by umbilical blood from the placenta to the fetus; in unpublished experiments, the author has found that this transport may be effected in part by the red blood cells. Last it may be noted that following parturition in women there sets in a momentary increase in the lipid content of the red cells.[5]

The lipid content of the red cells may be estimated directly by removing the cells from below the supernatant plasma after centrifuging the blood or indirectly from concurrent analyses of whole blood and plasma in conjunction with the hematocrit reading. Both of these methods are at present employed and frequently the results of one investigator using the direct method have been compared with those of another using the indirect. Hence, it was desirable to know which of these methods is the more exact and whether they gave different results.

The technic of the indirect consists simply in making the usual Bloor extract or other common extract of both whole blood and plasma. The technic of the direct method has been less thoroughly described. In doing direct analyses, the sample of oxalated blood is thoroughly centrifuged for about three-quarters of an hour or until the red cell layer has become translucent. This indicates that the interstices between cells have been displaced and that the cells are squarely packed and contain little or no plasma. Two cubic centimeters of these cells are pipetted off with suction if necessary and transferred to a 125 c.c. Erlenmeyer flask and the cells laked with a minimum amount of distilled water (about 1 c.c.). Then about 60 c.c. of alcohol-ether (3:1) are quickly poured in and the whole immediately and thoroughly shaken. The flask may have to be shaken again at short intervals to prevent clumping of the precipitated proteins. If, in spite of this, clumping occurs, the adherent mass must be removed, ground in a cleaned mortar with cleaned, sharp sand and then quantitatively transferred back to the original flask. Extraction is incomplete unless the precipitate is finely divided. The remainder of the extraction and analysis may be performed as previously described for plasma.[2, 4]

In this manner the lipid composition of the red blood cells was determined directly and indirectly in 10 normal men, collecting the specimens of blood under fasting conditions. The results obtained have been depicted in Table I in the form of means with standard deviations. The standard deviation was calculated by means of a formula previously used.[2] The percentage standard deviation was calculated by dividing the standard deviation by the mean and multiplying by 100. To conserve space, individual values will be summarily described rather than tabulated.

With almost every lipid the results were higher by the indirect than by the direct method. In 8 out of 10 cases neutral fat was higher, in 7 out of 10 the total fatty acids were higher, in 6 out of 10 the total cholesterol was higher, in 5 out of 10 the ester cholesterol was higher, in 7 out of 10 free cholesterol was higher, and in 6 out of 10 phospholipid was higher. In most of the cases where the results were lower by the indirect method the differences were slight, while when they were higher they were usually considerably so. As a result practically all the mean values were higher by the indirect than by

the direct method. It is thus not permissible to compare relative lipid values, some done by the direct and some by the indirect method of analysis.

The direct method was finally adopted in preference to the indirect after a consideration of the percentage standard deviations as listed in Table I. It may be noted that the percentage standard deviations were higher, sometimes by as much as three times, with the indirect than with the direct method. This signified that there was much greater variation among values obtained by the indirect method, a fact which was quite apparent from tables listing the individual values. Since values obtained by the indirect method are calculated from at least three sets of analytical figures, the experimental error involved is probably quite sufficient to account for this variation.

Further evidence in support of the superiority of the direct method was the fact that several "impossible values" were obtained by the indirect method and none by the direct. That is to say, the sum of the values for free cholesterol and ester cholesterol was greater than that of total cholesterol in three instances by the indirect method. In two other instances the total fatty acid value was less than the calculated sum of the individual fatty acids. As a result of these considerations, it was concluded that the direct method is to be preferred in the determination of the lipid composition of the red blood cells. This conclusion applies not only to the lipids in toto but to any one individual lipid such as total cholesterol.

TABLE I

A STATISTICAL COMPARISON OF VALUES FOR LIPIDS IN THE RED BLOOD CELLS AS DETERMINED BOTH BY THE DIRECT AND BY THE INDIRECT METHODS IN 10 NORMAL MEN
(EXPRESSED IN MG. PER 100 C.C.)

LIPID	MEAN OF 10 CASES		STANDARD DEVIATION		PERCENTAGE STANDARD DEVIATION	
	DIRECT METHOD	INDIRECT METHOD	DIRECT METHOD	INDIRECT METHOD	DIRECT METHOD	INDIRECT METHOD
Total fatty acids	275	297	37	117	14%	40%
Neutral fat	27	54	31	80	115%	148%
Total cholesterol	150	158	19	32	13%	20%
Ester cholesterol	28	27	20	23	71%	85%
Free cholesterol	124	138	18	28	14%	20%
Phospholipid	356	424	47	145	13%	34%

SUMMARY

The lipid composition of the red blood cells of ten normal men was estimated directly and indirectly by oxidative micromethods. In the majority of instances higher values were obtained by the indirect than by the direct method. The experimental error was found much greater by the indirect method which also gave more mathematically impossible values. Hence, it was concluded that the direct method is to be preferred in the determination of any one or all of the lipids present in the red blood cells, and that results obtained by one method cannot be compared with those obtained by the other.

REFERENCES

1. Bloor, W. R.: Fat Assimilation, J. Biol. Chem. **24**: 447, 1916.
2. Boyd, E. M.: A Differential Lipid Analysis of Blood Plasma in Normal Young Women by Micro-Oxidative Methods, J. Biol. Chem. **101**: 323, 1933.

3. Boyd, E. M.: The Lipemia of Pregnancy, J. Clin. Investigation **13**: 347, 1934.
4. Boyd, E. M.: Diurnal Variations in Plasma Lipids, J. Biol. Chem. **110**: 61, 1935.
5. Boyd, E. M.: Blood Lipids in the Puerperium, Am. J. Obst. & Gynec. **29**: 797, 1935.
6. Boyd, E. M.: The Lipopenia of Fever, Canadian M. A. J. **32**: 500, 1935.
7. Boyd, E. M.: Anaesthesia and the Blood Lipids, Surg. Gynec. Obst. **62**: 677, 1936.
8. Boyd, E. M.: The Effect of Pregnancy and Pseudopregnancy Upon the Blood Lipoids of Rabbits, J. Physiol. **86**: 250, 1936.
9. Boyd, E. M., and Wilson, K. M.: The Exchange of Lipids in the Umbilical Circulation at Birth, J. Clin. Investigation **14**: 7, 1935.
10. Boyd, E. M., and Tweddell, H. J.: The Lipids of Human Blood, Trans. Royal Soc. Canada, Section V **29**: 113, 1935.
11. Boyd, E. M., and Fellows, M.: Blood Lipids During Pregnancy in Guinea Pigs, Am. J. Physiol. **114**: 635, 1936.
12. Wendt, H.: Relation Between Phosphatides and Cholesterol in Whole Blood, Plasma and Erythrocytes of Healthy Men Overdosed With Olive Oil With and Without Previous Phosphate Administration, Biochem. Ztschr. **250**: 212, 1932; ref. Chem. Abstr. **26**: 5139, 1932.

BLOOD CHOLESTEROL AND THE MANIC DEPRESSIVE PSYCHOSIS*

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CHOLESTEROL is one of the most important constituents of cells, particularly cells of the central nervous system. It is important because it can, and does, exert much influence upon cellular metabolism, and, therefore, upon body metabolism in general. Although it has received much attention in many physiologic and pathologic conditions, it has been grossly ignored and neglected in mental disease. This is rather astonishing when one considers the fact that nervous tissue possesses an abundance of this chemical substance and that exclusive of its water content the brain is composed of about one-half lipoids of which over 25 per cent is cholesterol. With cholesterol composing the brain to this extent, neglect of its study, particularly in relation to mental disease, is almost heresy.

This paper is the preliminary report of a study of the blood cholesterol in one type of mental disease, the manic depressive psychosis.

In it will be considered the relationship existing between this chemical substance and the manic depressive psychosis, the manic phase, the depressive phase, and the state of mental and physical activity of the individual.

SURVEY OF LITERATURE

Pighini[1] studied seven cases of manic depressive psychosis and found the blood cholesterol to be increased. Tsuchiya[2] in his cases obtained only normal values. In the four cases of melancholia with anxiety examined by Targowla, Badonnel and Berman[3] the blood cholesterol was reported as high. Parhon and Parhon[4] stated that in their four cases of melancholia the values were below normal. In Ornstein's[5] cases the blood cholesterol was increased; in

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Jacobi's[6] cases it was decreased; and in Stenberg's[7] fourteen cases the blood cholesterol was definitely above normal. The average blood cholesterol in the six manic depressive cases reported by Gibbs[8] was 162.7 mg.; in the five depressions reported by Slight, Long and Salter[9] it was 239 mg.; and in the manic depressive and involutional melancholia cases reported by Duncan[10] it was 199 mg. and 216 mg., respectively. The literature on the relationship of blood cholesterol to this psychosis is thus obviously inadequate and conflicting. Further work is certainly needed to clarify this field and to stabilize the facts therein.

PROCEDURE

The individuals used for this work were established clinically as manic depressive. In each individual there was no demonstrable pathology other than the psychosis. This type of restriction was adhered to because there are certain pathologic states which of themselves would produce hypercholesterolemia or hypocholesterolemia, and it was felt that should individuals with pathology other than the psychosis be admitted to the group the results would not be reliable. From each individual was drawn by venipuncture, under sterile conditions, 5 c.c. of blood which was placed in a sterile oxalate tube, gently mixed with the oxalate, and placed in an ice box at 10° C. Tubes containing clotted blood were discarded. The blood for the controls was obtained from individuals, not patients, who were normal mentally and physically. The method used to estimate the blood cholesterol was a modified Myers-Wardell.[11] Duplicate estimations were made on each individual and the average used in each instance.

The mental and physical activity was evaluated thus: *Mental Activity:* State of cerebration, intellectual, emotional, and spatial. *Increased:* Irritability, quarrelsomeness, flight of ideas, distractability, agitation, active depression, active confusion and perplexity, active delusions and hallucinations. *Decreased:* Mental stupor, poverty of, or decreased cerebration. *Physical Activity:* State of bodily orientation with respect to a given point in space. *Increased:* Restlessness, active mannerisms, running, fighting, etc. *Decreased:* Inactivity, physical stupor, physical apathy, or depression.

RESULTS

This study consists of 71 cases of manic depressive psychosis and 53 normal individuals. The distribution of blood cholesterol values is shown in Table I. The range of the normal group is 110 to 195 mg. with a mean value of 148 ± 1.7 mg. and median value of 150 mg. There are 20 (37 per cent) cases below the normal mean and 34 (62.9 per cent) above the normal mean. The range of cholesterol values in the manic depressive group is 50 to 428 mg. with a median value of 104.6 mg. The mean value is not computed due to the unbalanced distribution of blood cholesterol values. In this group there are 40 (56 per cent) cases with blood cholesterol values below normal limits, 14 (19.6 per cent) cases above and 17 (23.8 per cent) cases within normal limits. There are 49 (68.6 per cent) cases below the normal mean and 21 (29.4 per cent) above the normal mean. From these figures it is quite

evident that only slightly less than one-fourth (23.8 per cent) of a total of 71 manic depressive individuals had blood cholesterol values within normal limits. As a group the manic depressive cases, therefore, present rather overwhelming evidence of abnormal cholesterol metabolism.

TABLE I

DISTRIBUTION OF BLOOD CHOLESTEROL VALUES IN NORMAL, MANIC AND DEPRESSIVE CASES. INDIVIDUALS GROUPED IN 10 MG. INTERVALS

NORMAL VALUES	NO. CASES	MANIC VALUES	NO. CASES	DEPRESSIVE VALUES	NO. CASES
110-120	3	50-60	1	50-60	1
120	4	60	1	70	2
130	6	70	2	80	4
140	8	80	3	90	3
150	11	90	9	100	2
160	10	100	12	110	1
170	5	110	2	120	1
180	5	120	1	140	4
190	1	140	1	150	3
Total	53	160	1	160	3
			33	200	2
				210	2
				220	2
				240	1
				250	1
				260	1
				280	1
				300	1
				320	1
				330	1
				420	1
					38

If the manic (33 cases) and the depressive (38 cases) groups are studied separately a similar although definitely altered picture is seen. In the manic group 28 (84.8 per cent) of the cases have cholesterol values which are below the limits of normal, and 5 (15.1 per cent) cases within it, and 5 (15.1 per cent) cases above it. In the depressive group there are 12 (31.5 per cent) cases with blood cholesterol values below normal, 12 (31.5 per cent) cases within normal limits, and 14 (36.8 per cent) cases above normal limits. The median value for manic cases is 100.4 mg. and for the depressive, 150 mg. It is, therefore, obvious that in the manic state there is a definite tendency for the blood cholesterol values to be decreased and below normal, whereas in the depressive cases there exists a fairly balanced tendency for the values to be not only within normal limits but below and above it in the same proportion. Briefly, 84.8 per cent of the manic cases and 68.3 per cent of the depressive cases have abnormal blood cholesterol values. This, too, is evidence of abnormal metabolism of a chemical substance which constitutes approximately 25 per cent of the lipoids of the brain.

To complete the study the relationship between the blood cholesterol and the state of the mental and physical activity was examined (Table II). When the physical activity alone is considered, whether increased (36 cases) or

decreased (24 cases), the median is quite low (98.5 and 110). Where the physical activity is increased, 62 per cent of the cases have blood cholesterol values below normal and 5.4 per cent of the cases have values above. Where the physical activity is decreased, 49.9 per cent of the cases have values below normal and 29.1 per cent above.

TABLE II*

CORRELATION OF BLOOD CHOLESTEROL AND TYPE OF MENTAL AND PHYSICAL ACTIVITY

	P. I.	P. D.	M. I.	M. D.	P. I. & M. I.	P. D. & M. D.	P. I. & M. D.	P. D. & M. I.	P. N. & M. I.	P. N & M. D.
Median	98.5	110.0	110.0	115.0	105.0	115.0				
Per cent below N	62.1	49.9	65.7	48.5	64.3	47.3	56.8	60	75	42.6
Per cent within N	29.7	20.8	26.3	21.2	32.1	21.0	28.4	20	–	14.2
Per cent below N mean	78.3	62.4	78.9	60.6	78.5	57.8	85.2	80	75	42.6
Per cent above N	5.4	29.1	7.9	30.3	3.6	31.5	14.2	20	25	42.6
Total cases	36	24	38	33	28	19	7	5	4	7

*Abbreviations used: P, Physical activity; M, mental activity; I, increased; N, normal; D, decreased.

In those cases where the mental activity is increased (38 cases) or decreased (33 cases) and considered alone, the median is low (110 and 115). Where the mental activity is increased, 65.7 per cent of the cases have blood cholesterol values below normal and 7.9 per cent above; where the mental activity was decreased, 48.5 per cent are below normal and 30.3 per cent above.

In those cases where both the mental and physical activity are increased (29 cases) or decreased (19 cases), the median value of the blood cholesterol is low (105 and 115). Where both the mental and physical activity are increased, 64.3 per cent of the cases have blood cholesterol values below normal and 3.6 per cent above. Where both mental and physical activity are decreased 47.3 per cent of the cases have blood cholesterol values below normal and 31.5 per cent above.

There are seven cases where the mental activity is decreased and the physical activity increased. Of these, 56.8 per cent have blood cholesterol values below normal and 14.2 per cent above. There are, also, seven cases where the mental activity is decreased and physical activity normal. Of these, 42.6 per cent have blood cholesterol values below normal and 42.6 per cent above. In four cases where the mental activity is increased and the physical activity is normal, 75 per cent have blood cholesterol values below normal and 25 per cent above. And in five cases where the mental activity is increased and the physical activity decreased, 60 per cent are below normal and 20 per cent above.

Therefore, in the manic depressive psychosis characterized by increased physical or mental activity or both, there is a definite tendency to an accompanying low blood cholesterol. Where there is an increased physical state accompanied by a decreased mental state there is a definite tendency for an accompanying low blood cholesterol. Where the psychosis is characterized by a decreased physical or mental activity or both there is a less definite tendency for an accompanying low blood cholesterol, but rather a tendency

for the values to be above and in the normal range. These findings are in accordance with the observations where, in the manic depressive group as a whole the median is definitely low, in the manic group the median is definitely low, and in the depressive group the median indicates a range of values, low, normal, and high.

These results may be questioned on the basis of the possible existence of factors other than the psychosis which may have been present to alter the blood cholesterol. Since the literature indicates that the blood cholesterol is increased in manic depressive psychoses, an attempt was made to determine whether pathologic entities other than the psychosis were present in the manic depressive cases. The results of this part of the study were entirely negative. No relationship could be demonstrated between blood cholesterol and clinically observed body type (medians: Pyknic 105.7 mg.; asthenic 103.3 mg.; and athletic 110.0 mg.) or state of abnormal nourishment (medians: obese 110 mg.; and undernourished 120 mg.). Albumin, indican, or acetone in the urine of some of the individuals apparently had no significance that could be demonstrated relative to the cholesterol. No relationship could be established between abnormal blood cholesterol and hemoglobin, erythrocyte count, or leucocyte count; nor could any relationship be established correlating blood cholesterol and systolic, diastolic, or pulse pressures.

DISCUSSION

The cases of manic depressive psychosis presented in this study can be divided roughly into three groups, viz., those with blood cholesterol (a) below, (b) above, and (c) within normal limits. For the purpose of this paper the cases with a normal value can be ignored, except for the recognition of the fact that a certain number of cases (23.8 per cent) *do* have a blood cholesterol within normal limits. This leaves 76.2 per cent of the cases with abnormal values to be explained. This 76.2 per cent automatically subdivides itself into those cases with values above normal (19.6 per cent), and those with values below normal (56 per cent).

Three-fourths of the cases of manic depressive psychosis have an abnormal blood cholesterol. And of these cases almost three-fourths have a blood cholesterol below normal.

The number of cases with an abnormal blood cholesterol and the distribution of this abnormality can hardly be due to chance. Since they must have some significance, the question naturally arises as to what it is that produces the abnormal blood cholesterol in these cases. At best, the most that can be done to answer the question at the present is to examine the possible sources whereby an abnormal value is obtained.

An increase in the blood cholesterol may be caused by (a) chronic liver disease, (b) diabetes mellitus, (c) arteriosclerosis, (d) lipoid nephrosis, (e) glomerulonephritis, and (f) pregnancy. Inasmuch as these conditions are not present in any of the cases, they cannot be held responsible.

A decrease in the blood cholesterol can be caused by (a) anemia, (b) inanition, (c) infection, and (d) acute hepatic disease. Since these conditions are not present in any of the cases they, too, cannot be held responsible.

In addition to these conditions which can alter the blood cholesterol, there are to be considered the endocrine glands, the autonomic nervous system, and the emotions. Although no clinical evidence of an abnormality of the first two is present, alterations in the endocrine and autonomic nervous system can exist without the presence of a measurable clinical syndrome. That the endocrine and autonomic nervous systems can alter the blood cholesterol has been shown many times both clinically and experimentally. And there is adequate evidence in this and the other papers[1-10] that altered emotions are accompanied in many instances by abnormalities in the blood cholesterol. Likewise it is well known that there is a close alliance between the endocrine glands, the autonomic nervous system and the emotions and that an alteration of one of these can affect profoundly the other two. It is very possible that the abnormality in the blood cholesterol in the manic depressive psychosis, is, to a certain extent, due to some imbalance of this triad. More cannot be said at this time, but further work in this field, it is hoped, will clarify the situation.

SUMMARY

1. A study of the blood cholesterol in the manic depressive psychosis is presented.

2. Abnormal blood cholesterol values are found in 76.2 per cent of the cases.

3. There is definite evidence that there is some correlation between the state of mental and physical activity and blood cholesterol.

REFERENCES

1. Pighini, G.: Ueber die Menge Cholesterins und Oxycholesterins des Serums bei verschiedenen Formen von Geisteskrankheiten, Ztschr. f. d. ges. Neurol. u. Psychiat. **4**: 629, 1910-11.
2. Tsuchiya, S.: Über Cholesterin in der Cerebrospinalflüssigkeit, Ztschr. f. Neurol. u. Psychiat. **90**: 255, 1924.
3. Targowla, R., Badonnel, M., and Berman, A.: Cholesterolemia in Mental Diseases, Encephale **18**: 138, 1923.
4. Parhon, C. J., and Parhon, M.: Cholesteremia in Mental Affections, Encephale **20**: 48, 1925.
5. Ornstein, I.: Cholesteremia in Insane, Compt. rend. Soc. de biol. **93**: 1622, 1926.
6. Jacobi, W.: Cholesterinstoffwechsel und Konstitutionspathologie der Psychosen, Monatschr. f. Psychiat. u. Neurol. **62**: 296, 1926.
7. Stenberg, S.: Psychosis and Blood Lipoids. Quantitative Variations of Total Cholesterin and Total Fatty Acids in the Blood. 1. Manic Depressive Psychosis, Acta. med. Scandinav. **71**: 559, 1929.
8. Gibbs, C. E.: The Suprarenal Cortex and Blood Cholesterol in Dementia Praecox, Am. J. Psychiat. **5**: 189, 1925.
9. Slight, D., Long, C. N. H., and Salter, R. W.: Plasma Lipoids in Mental Depression, Am. J. Psychiat. **13**: 141, 1933.
10. Duncan, A. G.: Serum Cholesterol in Mental Disorders, J. Ment. Sc. **76**: 284, 1930.
11. Schube, P. G.: A Method of Measuring Cholesterol, J. Lab. & Clin. Med. **18**: 306, 1932.

ALBUMINURIA SOLARIS*

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AMONG the various causes responsible for the anephritic albuminurias, there still are important sources hitherto unknown or unpublished.

Albuminuria, as is well known, is not identical with nephritis, the same as glycosuria is not identical with diabetes. Most of the anephritic albuminurias are innocuous in character and usually transient in appearance. It is well to point out the existence of an occasionally observed analbuminuric nephritis (per analogiam aglycosuric diabetes), in contrast to the anephritic albuminuria.

As an illustration, classifying the heterogenicity of the various types of albuminurias, a quotation of the well-known textbook of Herman Strauss, entitled *Die Nephritiden*‡ may suffice. Strauss makes reference to the following types of albuminurias: "accessory, accidental, alimentary, anaphylactic, constitutional, continuous, cyclic, ephemeric, extrarenal, fugax, hematogenic, innocuous, intermittent, lordotic, minimal, neurogenic, orthostatic, periodic, persistent, physiologic, renal, renopalpatory, spurious, toxic, transitory, traumatic, tubulogen"; furthermore he speaks of albuminuria in: "anemic conditions, apoplectic insults, bacteriotoxic states, cachexias, cancer, delirium tremens, diabetes mellitus, dispositions, Graves' disease, heart affections, icteric conditions, infectious diseases, metabolic disorders, passive congestions, thoracocompressions, traumatism (microtraumatism due to phosphaturia, oxaluria, uricaciduria)"; also during "pregnancy, parturition and puerperium; postepileptic, premenstrual, pretuberculotic; under emotional influences; in vagotonic conditions; after cold bath; after a general cold, after fatigue or physical strain, after marching or various sports or standing."

Under the term of *"albuminuria solaris"* we would designate transient albuminurias caused by skin reactions resulting from the exposure to sunrays. This albuminuria is *not* a febrile albuminuria. Its occurrence is not bound to the presence of fever; its precondition is a strong skin reaction only.

Under a sudden and unaccustomed exposure to sunrays, as is generally known, severe skin reaction will set in, but outside of the various degrees of burns on the skin exposed to the rays, general systemic reactions, such as chills, high temperature, general malaise, gastrointestinal symptoms, extensive herpes, etc., may follow. If albuminuria is observed under such conditions, this may range with the "albuminuria febrilis." Fever from any cause is followed in the overwhelmingly largest proportion of the cases by the appearance of albumin in the urine, called albuminuria febrilis, a condition,

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characterized by a mild, transient albuminuria of the nephrosis type, persisting during the feverish period, without the appearance of red blood cells or of casts in the sediment, or without any noticeable impairment of the kidney function. Dermatitis solaris with fever may correctly be classified in this group. However, in albuminuria solaris albumin is detected in the urine also in the "afebrile" cases of the dermatitis solaris; in other words albuminuria will be noticed in cases with milder skin reactions, when general symptoms are hardly noticeable and, in particular, fever is absent. Albuminuria undoubtedly serves, under such conditions, as a more sensitive accompaniment than the other generalized symptoms. In cases of albuminuria solaris albumin is detectable in the urine as long as new large areas of the skin are involved, characterized by the classical calor, turgor, rubor, dolor, and functio lesa, even though these syndromes are not strong enough to produce any noticeable fever or any other more marked general systemic reactions.

The albuminuria lasts as a rule as long as new large skin areas are involved, usually for a few days, rarely longer than a week. As soon as the acuteness of the process fades away, the redness subsides, even prior to the peeling of the skin or brownish pigmentation, the albuminuria disappears.

After one exposure to the sun, when the course of the albuminuria can best be seen and studied, one will notice that the albuminuria reaches its height after twenty-four hours to the exposure to the sun, and will remain unchanged for another twenty-four or thirty-six hours. Two or three days after the exposure, the albumin diminishes, until in another day or two it disappears entirely.

According to the degree of the burns of the skin, the amount of the albumin may be quite noticeable, in fact, considerable, ranging around a level of a ¼ or ½ pro mille, producing more than heavy traces, still with a negative finding in the centrifuged sediment. The albumin can be demonstrated with any of the usual methods (boiling of the acidified urine, the sulphosalicylic acid test, or Esbach's reaction, etc.).

The renal effect of the acute skin reaction, due to exposure to strong sunrays, is quite striking, the relative severity of the albumin reaction being out of proportion to the general systemic effect.

A few illustrative cases demonstrating the presence, frequency, uniformity and course of the albuminuria, simultaneously describing the way of its first detection, should be recorded.

A patient, A. D., fifty, white, male, who used to report for a periodic health examination twice a year, and whose urine in the past proved always to be free of albumin, happened to report for a routine office examination on one of the warmest days of July in 1935, incidentally after his first seashore sunbath in that year. Although the acuteness of the skin reaction was quite conspicuous, there was no fever or general malaise, and the skin was free of blisters. The physical examination was negative, except for a very definite albumin reaction in the urine, a finding unexpected and unexplained. The centrifuged sediment was entirely negative. Patient was ordered to bed, put on a strict diet and a specimen of urine was requested for the following day, for examination.

The specimen was brought to the office by the wife of the patient. She had a striking sunburn, the result of a similar sun effect, being in sharp contrast to her blond hair. The

color contrast involuntarily associated the idea of a possible coincidence between the sun effect and albuminuria, and she was asked to void some urine. To our surprise, the specimen showed, similarly, a definite albumin reaction, the same as her husband's.

The "experimentum crucis" of the test was then easy to produce. The couple had two children, fourteen and seventeen years of age, respectively, who were exposed to a similar sun effect, for the same length of time, and reacted with a similarly strong skin reaction. I requested a sample of urine from both of them, for immediate examination. They both contained albumin in identical amounts, in more than heavy traces. The centrifuged sediment was negative.

Thus four members of a family, being exposed under similar conditions, reacted with an equally strong skin reaction and exhibited albuminuria of a moderate, but marked, degree. Their regress and complete disappearance followed equally a uniform course. Urine specimens were received and examined daily. The maximal albumin reaction was found about thirty-six hours after the exposure. The albumin reaction of the following two days declined in intensity. After another day or two, about five or six days in all, the last albumin traces disappeared, in all the four specimens, equally.

The problem has, outside of its scientific significance, also some practical aspects. Without this knowledge, in many cases, when patients reporting for examination for any reason (to dispensary, to doctor's office, to insurance companies) during a hot July or August day, twenty-four or forty-eight hours after an exposure to a strong sun, and albumin was found in the urine, the latter would improperly be evaluated as to its classification or significance.

If, among others, H. S. Diehl and C. A. McKinley* found in a group of 20,000 young healthy men an incidence of albuminuria in 1,065 individuals (5.32 per cent), without the knowledge of albuminuria solaris, one cannot authoritatively state that the source of the unknown or unexpected albuminuria, at least in a certain percentage of the cases, might not have been explained in an eventual sun effect. This holds true of albuminurias of other etiology, where postural, emotional or any other cyclic or transient influences were found or supposed to exist.

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*Diehl, H. S., and McKinley, C. A.: Arch. Int. Med. 49: No. 1, 1932.

JUVENILE DIABETES MELLITUS*

A Study of Insulin-Like Reactions Unrelated to Hypoglycemia

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DURING observations on the juvenile type of diabetes mellitus over a period of fifteen years, the senior author has been impressed with the frequent incidence of a group of insulin-like reactions unassociated with hunger and unrelated to the amount of sugar or ketones in the urine or to the level of sugar in the blood.

These reactions have varied from a momentary lapse of consciousness to epileptiform convulsions resembling grand mal. It was found through routine examinations of the urine that they occurred simultaneously with an alkaline tide in the urine, and that they could be relieved readily by an acid-producing diet.

LITERATURE

The frequency of ketonuric acidosis in diabetes is too well recognized to be given special consideration here. The incidence of severe ketonuric acidosis in the nondiabetic has been especially well recognized in this country. This was described by Edsall[1] in 1903, Hecker[2] in 1911, and Marfan[3] in 1916 as cyclic vomiting and a disease of childhood. The severity and acuteness of onset and the rapidity of its subsidence are its outstanding characteristics. The infrequency of coma and the contrast of symptoms with those of diabetic ketonuria indicate a basic difference in the character of the acidosis. The literature is woefully lacking in pertinent pathologic data concerning cyclic vomiting. The authors' single postmortem observation showed extensive fatty degeneration of the liver with almost complete replacement of liver cells. There were no other findings.

It has been assumed that the acidosis of nephritis is due to the failure of the kidneys to excrete the acid products of normal metabolism, causing phosphoric acid retention. Marriott and Howland[4] in 1916 found a marked increase in the organic (acid) phosphate of the serum in nephritic patients with acidosis. Henderson and Palmer[5, 6] in 1915 found the ammonia (titratable acid) abnormally high. The incidence of nonketogenic acidosis in the diabetic may be aggravated by the underlying progressive degenerative lesions in the kidney tubule cells. The severity of this type of acidosis may be extreme, even fatal.[7-10]

HISTORY

In a careful review of the natural history of the juvenile diabetic, it was hoped that some relationship might be established between metabolic disturbances in earlier life and the onset of the diabetes. Because of the frequency of cyclic vomiting in diseases of children, detailed historic references were made

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This report is based on study of a group of forty case sof juvenile diabetes selected from the out-patient clinic at the Kings County Hospital. The patients were seen at regular intervals, at which time their weekly supply of insulin was replenished gratuitously.

to determine if such a relationship could be established and to trace it where possible. In this group, no patient gave a history of having had cyclic vomiting or any other serious metabolic upset prior to the incidence of his diabetes; nor could I find any such case in my private practice.

In the group, the most common childhood disease outside of measles was whooping cough, which occurred in 14 of the 40 cases. Thirty-three per cent of the males and 36 per cent of the females were Jews. In 2 of the cases there was direct family history of diabetes occurring in the father, and in 6 cases in the collaterals.

Obesity, with or without evident disturbances of fat metabolism, is a commonly observed prediabetic state of the diabetic patient of middle age. In the juvenile type of diabetes, herein considered, this relationship does not exist.

ENDOCRINE FACTORS

The sex development in the juvenile group seems to be well balanced. Three patients were diminutive and showed impaired growth. Two of these were given anterior pituitary extract in addition to insulin therapy with very little stimulation of growth. The tendency was toward lack of growth rather than excessive growth. The relation of the pituitary gland to the metabolism of the diabetic will be considered in a future article.

BLOOD CHEMISTRY

Blood sugar readings in the group studied reached as high as 400 and 500 mg. per 100 c.c. It is recognized that with dietetic regimen alone, blood sugar fluctuates most markedly.[11] There was no relationship between the height of the blood sugar and the reactions described.

The calcium and phosphorus determinations of the group ranged from a low of 8.3 mg. per 100 c.c. of calcium and 3.5 mg. of phosphorus to a high of 12.1 calcium and 3.14 phosphorus; and from a low phosphorus of 2.3 and 9.75 calcium to a high of 4.6 phosphorus and 11.1 calcium. The diabetic calcium-phosphorus ratio showed no variation from the normal or average readings.

INSULIN DOSAGE

The insulin preparations used were straight beef extracts, and the mixed extracts were obtained from beef, sheep, and pig. The mixed pancreatic extracts appeared to give more satisfactory results than the straight beef extract, but the preparations used seemed to bear no relation to the reactions under consideration. The dosage varied from a minimum of 20 units a day to a maximum of 141 units, the average daily dose being 61½ units. Of the group exhibiting reactions, one was receiving 20 units daily, one 44 units and the others over 50 units.

The occurrence of reactions unaccompanied by hypoglycemia has been described. In this group, there was no relationship between the brand and dose of the insulin. However, the possibility of some other factor in the insulin cannot be eliminated.

REACTIONS UNRELATED TO HYPOGLYCEMIA

The occurrence of reactions unaccompanied by hypoglycemia in the case of a woman aged fifty-nine years has already been described by the senior author.[12] Although insulin dosage ranged from 190 units to 400 units daily, there was persistent hyperglycemia with ketonuria and glycosuria. In this case, the insulin failed to bring the blood sugar down to a normal level at any time; yet there occurred reactions, at times accompanied by generalized twitchings and loss of consciousness, lasting as long as an hour. Obviously, the dosage of insulin was not an essential factor in the causation of these attacks.

Of the group reported, 16 cases demonstrated the reactions described, which ranged from a momentary lapse of consciousness to epileptiform convulsions. These reactions were not relieved by administration of sugar and were not associated with hypoglycemia; nor were they associated with skin manifestations, which would ordinarily occur in the event of foreign protein reactions.

PH OF URINE

Determination of the hydrogen ion concentration of the blood was considered unreliable and impractical in this group of cases. A simple method of determining the pH of the urine by the use of an indicator solution was adopted. This was made up and supplied to the individual patient, who was instructed as to its use. The following formula was used:

	Methyl red	20 mg.
	Bromthymol blue	40 mg.
	Ethyl alcohol	50 c.c.
Add		
	N/5 NaOH	2 c.c.
	Distilled water	150 c.c.
	Five per cent phenol	20 c.c.

Technic.—Float 6 drops of the test solution on half a test tube of urine. Color ring forming will be the same color as the urine for a neutral reaction, red for acid and green for alkaline reaction.

pH 7.5	Dark green
pH 7.0	Green
pH 6.5	Same color as specimen
pH 6.0	Red
pH 5.5	Dark red

Urines were tested several times during the day, but the highest point of alkalinity in these cases was reached in urines examined about 2 P.M.

TREATMENT

It was found that the alkaline tide in this group could be balanced by an acid-producing diet and that such a dietary correction resulted in relief of symptoms and reactions and simultaneous shift of the urinary reaction to the acid side. This dietetic treatment proved quite as effective as use of the ketogenic diet for idiopathic epilepsy in children. There were a few exceptions in cases

of patients who responded in the same way to administration of Fowler's solution, 3 minims twice daily.

In regard to dietary control, the author has always felt and impressed upon the juvenile diabetic that the diabetes is a maladjustment rather than a serious disease, and that the condition is amenable to adjustment of diet and the use of insulin. The diets were made ample and the carbohydrate-fat ratio of 3 to 1 was established.

The value of the low calorie diet is that of a stepping-stone to the work diet or permanent diet. The work diet, as the name implies, forms the basis of the active life diet of the individual.

REPORT OF CASES

Full details of the 40 cases studied are omitted because of space limitation. The following case reports have been selected as most typical of the group:

CASE 1.—White male, English, twenty-two years of age, 5 feet 7 inches tall, weight 154 pounds, had diabetes mellitus for four years. He was admitted to the hospital for middle ear infection, which cleared up spontaneously about two weeks after admission.

Father died at forty-two years of age of pulmonary tuberculosis, and the mother now has active tuberculosis. The only previous diseases were whooping cough, measles, and chickenpox. Physical examination of the patient was essentially negative, except that electrocardiography revealed a picture of sinus tachycardia and myocardial damage.

Ordered on a diet of: carbohydrates 60, proteins 50, fats 125, calories 1,600, with 30 units of insulin; but violated rules and ate the same as the rest of the family. A check revealed that the family ate about: carbohydrates 250, proteins 100, fats 120, calories 2,500. He was placed on this diet and 120 units of insulin, which maintained him with rapid gain in weight and strength. His blood sugar on admission was 410 mg. per 100 c.c. His fasting sugar was 213 mg. per 100 c.c.; and the blood taken two hours after breakfast, with insulin, was 150 mg. per 100 c.c.

On discharge from the hospital he complained of attacks of dizziness not relieved by administration of sugar. The pH of the urine at 11:30 A.M. was 7.8. The average for the week taken at 2 P.M. was 7.5.

The patient was placed on an acid carbohydrate diet of: carbohydrates 250, proteins 100, fats 120, calories 2,500, and has not complained of these symptoms since. When tested in the clinic, the urine pH at 11 A.M. was 7.0.

CASE 2.—White female, Irish, aged ten years, height 4 feet 7 inches, weight 75 pounds. The mother and father are living. The mother has pulmonary tuberculosis, and the father has chronic alcoholism.

The child was operated upon for a ruptured appendix in November, 1932. She has had scarlet fever, whooping cough, and measles.

In August, 1933, the patient suddenly developed polyuria, loss of weight, thirst, hunger, weakness, and finally coma. As soon as diagnosis of diabetes mellitus was made, she was placed upon a diet of: carbohydrates 200, proteins 100, fats 100, calories 2,100, and 44 units of insulin. She recovered quickly from coma.

In the early summer of 1935, the patient had six attacks of amnesia and was mentally confused every day for a period of time around the noon hour. This condition was quickly relieved by food. On July 4, 1935, she had an attack of convulsions. She suddenly began to scream and shriek while at lunch. This was followed by throwing the head to the right, general convulsions, cyanosis, frothing at the mouth, and unconsciousness, lasting forty-five minutes. This attack was not relieved by administration of sugar or any medication.

Following the attacks the urine was found to have a pH of 7.4 in the early afternoon. The patient was treated with an acid carbohydrate diet and 4 minims of Fowler's solution per day. The urine pH was found to be constantly 7.0. There were no further attacks.

The physical examination revealed atrophy of the muscles of both thighs and arms. This occurred at the site of injection of the insulin. The rest of the findings were essentially negative.

CASE 3.—(Previously reported.[13]) H. M., Jewish boy, developed diabetes in the summer of 1923, when he was nine years of age. There had been a gradual loss of strength and weight and pain in the epigastrium. He was treated with insulin and diet and urine became sugar free.

Unfortunately, he fell into the hands of a quack, upon whose advice insulin was stopped on Sept. 16, 1924, and a liberal diet was instituted. On September 18, patient was admitted to the hospital in a comatose state. He was given insulin, clyses, and blood transfusions. Improvement was progressive and rapid, and he was discharged from the hospital on September 23.

Dietetic regimen, on account of his low weight, was gradually changed. At the end of six months he was getting 2,000 calories: carbohydrates 200, proteins 85, and fats 100. Insulin was increased from 30 units daily to 22 units in the morning and 24 at night. Patient was perfectly well except for occasional insulin reactions, although he had never been entirely free from glycosuria. Because each attempt to increase the insulin content resulted in insulin shock, patient was permitted to run glycosuria, once or twice daily, but there was at least one clear specimen during the day.

He noticed while at college during the school year of 1931 that he was subject to temporary periods when there was a loss of memory. At these times his school notes were incomplete. The state of unconsciousness was momentary and lasted at the most from one to four seconds. The lapses occurred in the afternoon between one and three o'clock. Urinary examinations made from one to three hours after these spells were positive for sugar, usually showing a trace. Blood sugar determinations were made repeatedly at this time of the day and on no occasion revealed hypoglycemia.

On May 5, 1932, the character of the reactions changed and the patient developed definite epilepsy. Examinations of the urine immediately after this occurred were positive for sugar but alkaline in reaction. A second epileptic attack occurred on May 10, at which time urine examination also revealed presence of sugar and an alkaline reaction. Alkalinity was persistent for a number of days after this.

July 7, patient again had epileptic attack. Examination of urine showed sugar present, alkaline reaction. Van Slyke CO_2 was 76. August 15, patient had an epileptic attack. September 17, epileptic seizure. October 22, there was another epileptic attack. During intervals in this period, patient again noticed the short lapses of memory. December 1, epileptic seizure. Patient was placed upon an acid-producing diet and was given Fowler's solution. The insulin dosage was maintained and the diet was unchanged except for substitution of acid juices.

He has been seen at intervals for the past three years and has had no complaints. There have been no repetitions of epileptic attacks.

CASE 4.—D. B. was first observed in November, 1925. He was then two years and three months. He was 3 feet 2 inches tall and weighed 30 pounds. His family history was negative for diabetes. He was emaciated and complained of thirst and frequency of urination. Two months prior to illness he had had an attack of tonsillitis and during the three weeks prior to observation showed evidence of marked thirst and polyuria. Urine sugar was 3 per cent and blood sugar was 240 mg. per 100 c.c. Ketonuria was present.

He was placed upon a diet of carbohydrates 80, proteins 50, fats 56, and given 6 units of insulin morning and night. The diet was gradually increased and the insulin dosage was maintained.

October, 1927, the patient had enlargement of the liver. He felt well and was active. He had exhibited insulin shock reactions with this small dosage of insulin. April, 1930, weight was 40 pounds; height was 3 feet 3 inches. There was an increase of only 1½ inches in five years. At this time, his twin brother developed pneumonia and died three hours after onset.

In 1931, insulin dosage was increased to 8 units morning and night. His brother, only four and one-half years old, was taller and stronger. It was during this interval that the

patient developed periods of petit mal; urinary sugar was present during these attacks. They would occur at intervals of every two or three weeks. These attacks interfered with the child's attendance at school. Examination of urine at these times revealed an alkaline tide which persisted in spite of dietetic replacement. Fowler's solution was administered, 3 minims twice a day. The spells ceased after two weeks of administration. At this time the child was taken to Pittsburgh.

December, 1935, patient weighed 50 pounds and was 4 feet 1 inch tall. Mother reported that the child had had no spells as heretofore described and he was now being given the pituitary growth hormone. He was 4 feet 1 inch tall and weighed 50 pounds, while the younger brother was 4 feet 4 inches tall and weighed 60 pounds.

CASE 5.—Male physician, aged twenty-four years, developed diabetes at the age of nineteen years. It was discovered upon examination of the urine. He was 5 feet 5 inches tall and weighed 130 pounds. He had severe whooping cough when a child. His father had died of diabetes at the age of 51. Two brothers aged 26 and 27, respectively, had diabetes.

He was placed upon a diet of carbohydrates 260, proteins 80, fats 140, without insulin. Between 11 A.M. and 1 P.M. he observed reactions during which he would perspire freely and tremble. There was inability to concentrate. This was not associated with hunger.

During one attack, the blood contained 200 mg. of dextrose per 100 c.c. Urine examined at these times showed marked alkaline reaction ranging from pH 7.0 to pH 7.5. Diet was changed and acid-producing fruit substituted for the alkaline-producing, with complete alleviation of these symptoms and a change in the urinary reaction from alkaline to neutral and acid.

SUMMARY

In a series of 40 cases of juvenile diabetes mellitus, insulin-like reactions, unrelated to hypoglycemia, were observed in 16. In many cases the reactions resembled attacks of petit or grand mal. Of uniform occurrence in these cases was a definite alkaline tide, the alkaline pH of the urine reaching its peak about 2 P.M. An acid-producing diet proved remarkably effective both in bringing the reaction of the urine within the acid range and relieving the insulin-like reactions of epileptiform seizures. Administration of small doses of Fowler's solution often had a similar effect.

REFERENCES

1. Edsall, D. L.: A Preliminary Communication Concerning the Nature and Treatment of Recurrent Vomiting in Children, Am. J. M. Sc. **125**: 629, 1903.
2. Hecker, P.: Periodisches Erbrechen mit Acetonämie, Ergebn. d. inn. Med. u. Kinderh. **7**: 242, 1911.
3. Marfan: Diagnostic et traitement des vomissements périodiques avec acétonémie, Bull. méd. **30**: 605, 1916.
4. Marriott, W. McK., and Howland, J.: Phosphate Retention as a Factor in the Production of Nephritis, Arch. Int. Med. **18**: 708, 1916.
5. Henderson, L. J., and Palmer, W. W.: On the Several Factors of Acid Excretion in Nephritis, J. Biol. Chem. **21**: 37, 1915.
6. Palmer, W. W., and Henderson, L. J.: Clinical Studies on Acid Base Equilibrium and the Nature of Acidosis, Arch. Int. Med. **12**: 153, 1913.
7. Revillet: Coma chez une diabétique sans acétonuria, Lyon méd. **122**: 815, 1914.
8. Rosenbloom, J.: A Form of Diabetic Coma Not Due to the Acetone Bodies, New York M. J. **102**: 294, 1915.
9. McCaskey, G. W.: A Case of Fatal Diabetic Coma Without Diacetic or Betaoxybutyric Acid, J. A. M. A. **66**: 350, 1916.
10. Starr, P., and Fitz, R.: The Excretion of Organic Acids in the Urine of Patients With Diabetes Mellitus, Arch. Int. Med. **33**: 97, 1924.
11. Feinblatt, H. M.: Hyperglycemia—Based Upon a Study of 2,000 Blood Chemical Analyses, J. LAB. & CLIN. MED. **8**: 500, 1923.
12. Feinblatt, H. M.: Three Cases of Endocrine Disease With Pronounced Asthenia, M. Clin. North America **11**: 107, 1928.
13. Feinblatt, H. M., and Sherman, I.: Report of a Very Severe Case of Juvenile Diabetic Coma in Which Combined Treatment With Insulin and Blood Transfusion Resulted in Prompt Recovery, J. LAB. & CLIN. MED. **11**: 63, 1925.

616 CARLETON AVENUE

IDIOPATHIC AMYLOID DISEASE OF THE HEART*

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THOUGH "waxy" or "lardaceous" disease was recognized before Virchow's time, these terms referred to a variety of ill-defined conditions many of which would probably not be so classified today. He, it was, who in 1853 discovered that organs containing the substance which he named "amyloid," possessed a characteristic staining reaction with iodine and sulphuric acid.[1] Since that time numerous studies and case reports on amyloidosis have appeared in the literature, and the subject has been attacked from many different angles. In spite of these investigations, the exact nature of amyloid is still a matter of dispute,[2] but it seems certain that its composition varies in different cases and even in different organs.

Most observers agree that amyloidosis is now much less common than in the past.[3] This decrease they attribute to improvement in the treatment of the chronic suppurative diseases which, in some fashion still undetermined, frequently form the basis for its development. Some of this decrease is counteracted by the increased frequency of the clinical diagnosis made possible by the introduction of Bennhold's Congo red test. Not only has the diagnosis in doubtful cases been confirmed by the use of this test, but also it has been found that spontaneous regression may occur, as in the cases of Walker[4] and Reimann.[5]

In many instances, however, a suppurative focus is lacking, and the fault must be laid at the door of the associated disease, such as carcinoma, syphilis, leucemia, or myeloma. Though the presence of one or other of these conditions may afford an easy but unsatisfactory explanation of the etiology, occasionally no such background can be discovered. Idiopathic amyloidosis was known as early as 1856 when Wilks[6] reported several cases. Its infrequent appearance has nearly always been the occasion for interesting but fruitless speculation as to the cause.

Practically every organ in the body has been the site of amyloid deposits, though liver, spleen, and kidney are by far the commonest locations. In spite of the often widespread distribution of the process and the involvement of important organs, amyloidosis is uncommonly a cause of death per se. Usually the underlying disease is responsible for the fatal termination. In rare instances, though, an organ may be so extensively infiltrated that the failure of a vital function results. Most frequently this occurs in the kidneys, causing renal insufficiency.[7, 8] There have also been reported a few cases[9, 10] in which the adrenal tissue was almost wholly replaced by amyloid, the patients dying with the symptoms of Addison's disease.

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That amyloid deposits are found in the heart was known to Virchow.[1] From time to time cases are recorded, in which the heart is involved as part of a generalized process. Rare, however, are the instances in which myocardial amyloidosis ranks as the primary cause of death. In 1886, Wild[11] reported a case in which the patient, who suffered from erysipelas, presented the clinical picture of cardiac failure. At autopsy extensive deposits of amyloid were found throughout the heart. In the case of Steinhaus,[12] marked infiltration of the heart and gastrointestinal tract accounted for the symptoms of heart failure and intestinal upset. In 1926, Silwer and Lindblom[13] presented the case of a male of thirty-four years with symptoms of cardiac insufficiency for ten months. He showed marked signs of cardiac failure, and apparently died from this cause. Autopsy revealed amyloidosis of heart, liver, and spleen, but no etiologic factor could be demonstrated.

In Larsen's recent example,[14] the patient had symptoms of left-sided heart failure for about a year. In this case the amyloid deposits were located chiefly in heart, lungs, and kidneys, the liver and spleen being uninvolved. Perla and Gross[8] have described three cases of atypical amyloid disease, in one of which death was due to widespread replacement of cardiac muscle by amyloid. Reimann, Koucky and Eklund[15] recently studied amyloidosis in a patient who died of a terminal peritonitis. The organs containing amyloid were tongue, heart, lungs, esophagus, and pelvic organs.

Though the two cases, the details of which follow, possess certain clinical features in common, the differences in the pathologic findings are striking.

CASE 1.*—*Clinical History.*—The patient, a white female of fifty-one years, was admitted on March 13, 1927, to the Medical Service of the Toronto General Hospital under Professor Duncan Graham. She complained of feeling "run down" for about the past seven years with only occasional short periods of good health. For a year she had felt weaker, had lost 20 pounds in weight and suffered from an intermittent dry cough. These symptoms became progressively worse, particularly in the three weeks prior to admission, most of which time was spent in bed. She confessed to slight shortness of breath on exertion but denied any swelling of the feet, palpitation or precordial pain. Except for a moderate degree of constipation for many years, functional inquiry revealed no other complaints. No significant facts were elicited in the past history.

Physical Examination.—In appearance the patient was rather pale and undernourished. The heart was moderately enlarged to percussion; no murmurs were heard. The blood pressure was 76/48. There was no edema of the dependent parts. Examination of the chest revealed impairment of resonance and breath sounds at both lung bases; a few râles were heard in the left axilla. There were no other findings of importance.

Laboratory Record.—The urine had a specific gravity of 1.017, showed a faint trace of albumin and contained a few pus cells.

The hemoglobin was 88 per cent (Sahli); the red cells numbered 5 million per c.mm. and the white cells, 6,700. The blood smear was not remarkable and the differential white count was normal.

The blood Wassermann and Kahn tests were negative.

The electrocardiogram showed left ventricular preponderance and low voltage.

Roentgenograms of chest, gastrointestinal tract, gallbladder, and vertebral column were negative.

Clinical Diagnosis.—Chronic degenerative myocarditis; cardiac hypertrophy; myocardial insufficiency.

*This case is reported through the courtesy of Dr. J. E. Bates who performed the autopsy.

Though the patient showed no obvious change in her condition following admission, she died suddenly on the third day.

Autopsy Findings.—Slight pitting edema was demonstrable in the lower portions of the legs. Examination of the thorax showed both visceral and parietal layers of the pericardium to be studded with firm, translucent, grayish nodules, measuring up to 1 mm. in diameter. Similar deposits were likewise present on the pleura; some of these were large and plaque-like, measuring up to 0.5 cm. across. A few of these nodules were also found throughout the middle lobe of the right lung. The peritoneum was everywhere smooth and glistening. The mucosal surfaces of stomach, small and large intestine, and bladder, showed a diffuse scattering of small, gelatinous nodules similar to those seen on pericardium and pleura. The liver, spleen, and kidneys presented no gross evidence of amyloid disease.

Nowhere could any suppuration or other lesion be found to account for the amyloidosis.

Heart: There was marked enlargement affecting all chambers, the heart measuring 14 by 11.8 by 7 cm. The entire pericardial surface was studded with the readily palpable, grayish nodules described above. The myocardium was firm and waxy in consistency and offered increased resistance to the knife. The wall of the left ventricle measured up to 1.3 cm. in thickness, and that of the right up to 0.5 cm. The muscle was reddish brown in color and the cut surface was everywhere occupied by minute, glassy, gray areas many of which protruded slightly above the surface. Several portions of muscle were dipped in iodine. In a few moments these areas became mahogany brown in color, giving the surface a stippled appearance. Beneath the endocardium of all chambers were many fine shotty nodules. Similar deposits were present along the free margins and over the auricular surfaces of the tricuspid valve. Those on the mitral valve were fewer in number but distributed in like fashion. On the pulmonary valve these were wholly confined to the ventricular surface and on the aortic valve to the free margins. The great vessels presented no gross evidence of amyloid change.

Microscopic Examination.—Heart: There was extensive replacement of myocardium throughout by amyloid deposits which formed roughly about 60 per cent of the total area of heart muscle as seen in the sections. The distribution was both patchy and diffuse in character. Many of the patches were extremely large, some of them occupying several low power fields. In none of these were muscle fibers completely lacking, all of them containing a few, more or less isolated strands. They were quite irregular in shape and none was definitely delimited. All shaded off gradually into the less affected zones. No areas were uninvolved. Thin bands of amyloid penetrated between the individual muscle fibers. Many of the masses were small, rounded, and more or less sharply delimited. Some contained in their centers small venules which seemed narrowed and distorted; others showed no traces of blood vessels. All, however, formed an expanding mass which pushed aside and compressed the nearby muscle cells. The nodules on both the pericardium and endocardium were made up of irregularly lamellated masses of amyloid covered on their free surfaces by endothelium. The deposits on the valves were similar in appearance.

Sections stained with Sudan III showed a scattering of fine fat droplets distributed diffusely.

Lungs: The deposits of amyloid were found in the alveolar walls which were so greatly thickened that in some places groups of the alveoli were collapsed. The nodules on the pleura were similar to those on the pericardium. The peribronchial lymph nodes were almost completely replaced by dense, homogeneous amyloid containing a few collections of anthracotic pigment.

Gastrointestinal Tract: Everywhere the submucosa was occupied by small, irregular masses of amyloid, some of which surrounded and almost occluded the blood vessels.

No amyloid was present in liver, spleen, kidneys, or any other organs.

CASE 2.—*Clinical History.*—The patient, a woman fifty-nine years old, came under the care of Professor Duncan Graham at the Toronto General Hospital on Oct. 1, 1934. She had been well until four months previously, when she noticed that an unusual amount of walking caused slight swelling of her feet. At first this was present only late in the day, and was relieved by a night's rest. In the three weeks before admission, the swelling was present constantly, and it gradually increased in extent. About the same time as the onset of the

edema, she began to have shortness of breath on exertion which grew progressively worse. For two months her abdomen had been slowly increasing in size. No facts of significance were revealed in the functional inquiry or the past history.

Physical Examination.—The patient appeared quite ill, dyspnea being a prominent feature. Pitting edema was present over the whole body up to the level of the third ribs anteriorly. The legs were swollen and tense. The veins of the neck were engorged and pulsating.

The borders of the heart could not be percussed due to edema and tenderness of the chest wall. The heart rate averaged 100 per minute, the rhythm being normal. The blood pressure was 130/90. The abdomen contained free fluid.

Laboratory Record.—The urine had a specific gravity of 1.026 and showed albumin (3+) with an occasional hyaline cast and pus cell.

The blood smear and differential white count were not abnormal and the hemoglobin was 77 per cent (Sahli). The blood nonprotein nitrogen was 44 mg.

Unfortunately a blood Wassermann was not obtained.

The electrocardiogram revealed right axis deviation with a voltage = 2 mm.

Clinical Diagnosis.—Chronic degenerative myocarditis; myocardial insufficiency.

The day following admission, the patient's dyspnea grew worse, and the right chest was aspirated, 900 c.c. of clear, yellow, sterile fluid being removed. Later the same day, she developed acute pulmonary edema, successfully treated by atropine and venesection. The next day she rapidly became worse and died.

Autopsy Findings.—The legs were swollen and tense, and pitting edema was present over the body up to the level of the manubrium sterni. The right pleural cavity contained 600 c.c. of clear yellow fluid; and the left, 800 c.c. Both lung bases showed hypostatic pneumonia. About 600 c.c. of yellow fluid was obtained from the peritoneal cavity. The liver and kidneys were not remarkable in the gross. With the exception of the spleen, none of the abdominal organs gave a positive reaction with iodine.

No lesion could be found to form a logical basis for the development of amyloidosis.

Heart: The heart was slightly enlarged, measuring 12 by 10 by 6 cm., and was definitely heavier than normal, weighing 360 gm. The pericardium was smooth except for a small "milk spot" on the anterior surface. The myocardium felt firm and waxy and the cut surface was a pale, homogeneous, glassy, reddish brown color. Several portions were soaked in iodine and almost immediately the tissue became flecked with dark brown streaks and dots. The endocardium and valve cusps were smooth and glistening throughout.

Spleen: The spleen was large and heavy, measuring 11 by 7.5 by 5 cm. and weighing 270 gm. It was extremely firm in consistency, like hard rubber, and little impression could be made on it with the fingers. The cut surface was quite hard, bright rose red in color, and the normal markings were obliterated. On treating with iodine, the surface became a deep mahogany brown. The distribution was unusual, the staining being diffuse throughout. Attached to the hilus of the spleen were two small accessory spleens, the larger measuring 2.5 cm. in diameter. Their cut surfaces were similar in appearance and in consistency to the larger organ. Both turned a dark brown on the application of iodine.

None of the other organs presented any important gross abnormality and none reacted positively to iodine.

Microscopic Examination.—Heart: Sections taken from all portions of the heart showed extensive infiltration and destruction of muscle by amyloid. For the most part the lesions were diffuse in character. Between the individual muscle cells lay thin strands of pale fibrillar material which was stained a faint pink by Congo red. The small nodular masses so common in the previous case were seen infrequently. They were located chiefly in the fibrous trabeculae, and it is quite possible as Larsen[14] suggests, that these were all primarily derived from a blood vessel. The large patches which were not numerous appeared to have resulted from the disappearance of muscle and the coalescence of many small strands.

With respect to distribution, no part of the myocardium was free from the lesions. The ventricular muscle appeared to be affected to a greater degree than the auricular. The

interventricular septum showed the most extensive involvement of all. The papillary muscles were similarly infiltrated but with the exception of a few small patches in the aortic valve, none of the valves contained amyloid. Neither pericardium nor endocardium possessed the nodules so prominent in Case 1. The few deposits seen were flattened and related to contiguous masses in the underlying myocardium.

In Sudan preparations extensive fatty degeneration of muscle was noted, many of the fibers being almost obscured by myriads of tiny fat droplets. This condition was much more marked here than in the previous case.

The larger coronary vessels were uninvolved save for a few small areas in the adventitia. Some of the small arterioles showed a mild degree of sclerosis.

Aorta: No amyloid deposits were found.

Liver: There was marked alteration of structure due to extreme chronic passive congestion. Many of the liver cells had disappeared and the remainder were peppered with fine droplets of fat. The amyloid deposits were located chiefly in the outer portions of the walls of the large vessels. A number of small masses were seen in the portal areas with a few thin strands passing toward the central veins between the liver cords.

Spleen: The splenic pulp was diffusely infiltrated with amyloid. Many of the cells were completely destroyed and the rest were represented by pyknotic nuclei. Most of the malpighian corpuscles had disappeared and the remainder showed up as small collections of lymphocytes, each surrounding a compressed, distorted vessel. The accessory spleens presented the same picture.

Kidneys: A few patches of amyloid were seen in the walls of the larger vessels.

Adrenals: Both glands showed heavy amyloid deposits chiefly in the medulla and inner layers of the cortex.

None of the remaining organs revealed any traces of amyloid.

DISCUSSION

These two cases possess a number of features in common. Both occurred in middle-aged women whose past history was free from any chronic illnesses. In both, the symptoms suggested cardiac failure presumably from sclerotic heart disease, and the clinical and laboratory findings supported this diagnosis. Both apparently died of heart failure. In neither could be found any adequate explanation for the development of amyloidosis. The hearts were enlarged, though unfortunately a strict comparison cannot be made as there is no record of the weight in the first case. Larsen[14] stated that all cases reported previous to his had had atrophic hearts. Since then, Budd,[16] and Perla and Gross[8] have recorded cases in which the heart was hypertrophied.

Considerable difficulty was experienced in obtaining characteristic staining reactions in paraffin sections of the heart. With Congo red the amyloid stained in a pale and patchy fashion. Much better results were seen in sections of spleen and liver in the second example. The fact that in atypical amyloidosis there may be failure of the usual reactions was emphasized by Lubarsch.[17] Another possibility is suggested by the experiments of Smetana,[18] who found that recently formed amyloid showed up only after intravenous injection of Congo red.

A number of important variations were also noted. The distribution of the lesions outside of the heart was quite different. In the first case these were found in lungs, pleura, and submucosa of gastrointestinal tract and bladder; in the second, the spleen, liver, and adrenals were affected.

Then, too, the characters of the heart lesions differed. In the one the pericardial and endocardial surfaces were studded with nodules of amyloid; in the other these surfaces were grossly uninvolved. The myocardial lesions were both nodular and diffuse in the first, but in the second, diffuse only. Whether any significance can be attached to this difference is uncertain. Is this type of nodular deposit, as Lubarsch[17] suggests, frequently present in atypical amyloidosis? Is there any relationship between their absence in the second case and the involvement of spleen and liver? These questions can only be answered by further research on the mode of amyloid formation.

Larsen[14] has described in detail the formation and spread of cardiac deposits. He believes that they always arise from a pericapillary nidus and extend into the interstitial tissue, surrounding the muscle fibers and cutting off nutrition. Occasionally invagination and penetration of the individual fibers occurs. In both of our cases the muscle cells showed marked fatty degeneration which was probably due to failing nutrition and represented a stage in the destruction of the cell. To what degree death was due to replacement by amyloid and to what degree it was due to the fatty degeneration of the remaining muscle, it is not possible to say. In any case, amyloidosis was the basic factor.

The classification of myocardial amyloidosis has been attempted by a number of authors. The simplest method is to divide the cases into two groups as does Larsen:[14] those in which the heart is affected as part of a widespread process, and those in which it is involved usually to the exclusion of the commonly affected other organs. Still a third class is recognized by Budd,[16] those in which there is generalized disease of the muscle systems. It is questionable whether such classifications serve a useful purpose other than to bring to mind the variety of distribution found. While the first case would fall into the former class, the second combines the features of both classes and bridges the gap between the two.

With respect to etiology these cases present nothing that is new. Susman[19] suggests that, of the substances which produce amyloidosis experimentally, the common factor is toxicity or the ability to damage tissues, and that the spleen in some way is necessary to the process although the formation is of local manufacture. This is not wholly inconsistent with the results of Smetana's[18, 20] experiments which demonstrated the important rôle of the reticulo-endothelial system in amyloid formation. In Warren's[21] example of amyloidosis of the muscles, he attributed it to "a widespread perversion of fibroblastic function." The findings in the above cases do nothing to refute either contention.

SUMMARY

1. Two cases of idiopathic myocardial amyloidosis are described.

2. The various pathologic features are discussed and compared with previously reported cases.

REFERENCES

1. Virchow, R.: Cellular Pathology (translated by F. Chance), American ed. 7, New York, 1858, Robert M. De Witt, p. 409.
2. Wells, H. G.: Chemical Pathology, ed. 5, Philadelphia, 1925, W. B. Saunders Co., pp. 469-475.

3. Rosenblatt, M. B.: The Clinical Manifestations of Amyloidosis, Ann. Int. Med. **8**: 678, 1934.
4. Walker, G. F.: Case of Recovery From Amyloid Disease, Lancet **2**: 120, 1928.
5. Reimann, H. A.: Recovery From Amyloidosis, J. A. M. A. **104**: 1070, 1935.
6. Wilks, S.: Cases of Lardaceous Disease and Some Allied Affections. Guy's Hosp. Rep. **2**: Series 3, 105, 1856.
7. Dixon, H. M.: Renal Amyloidosis in Relation to Renal Insufficiency, Am. J. M. Sc. **187**: 401, 1934.
8. Perla, D., and Gross, H.: Atypical Amyloid Disease, Am. J. Path. **11**: 93, 1935.
9. Hunter, W. C., and Rush, H. P.: Amyloidosis of Adrenals as Cause of Addison's Disease, Ann. Clin. Med. **5**: 404, 1926.
10. Philpott, N. W.: Addison's Disease in Association With Amyloidosis, Ann. Int. Med. **1**: 613, 1928.
11. Wild, C.: Beitrag zur Kenntnis der amyloiden und der hyalinen Degeneration des Bindegewebes, Beitr. z. path. Anat. u. z. allg. Path. **1**: 175, 1886.
12. Steinhaus, F.: Ueber eine seltene Form von Amyloid- und Hyalininfiltration am Cirkulations- und Digestionsapparat, Ztschr. f. klin. Med. **45**: 375, 1902.
13. Silwer, H., and Lindblom, A. F.: Ein Fall von allgemeiner Amyloidose ohne nachweisbare Ursache, Acta med. Scandinav. **64**: 529, 1926.
14. Larsen, R. M.: A Pathological Study of Primary Myocardial Amyloidosis, Am. J. Path. **6**: 147, 1930.
15. Reimann, H. A., Koucky, R. F., and Eklund, C. M.: Primary Amyloidosis Limited to Tissue of Mesodermal Origin, Am. J. Path. **11**: 977, 1935.
16. Budd, J. W.: Primary Amyloid Disease of the Heart, Am. J. Path. **10**: 299, 1934.
17. Lubarsch, O.: Zur Kenntnis ungewöhnlicher Amyloidablagerungen, Virchows Arch. f. path. Anat. **271**: 867, 1929.
18. Smetana, H.: Experimental Study of Amyloid Formation, Bull. Johns Hopkins Hosp. **37**: 383, 1925.
19. Susman, W.: Amyloidosis, Edinburgh M. J. **34**: 527, 1927.
20. Smetana, H.: The Relation of the Reticulo-Endothelial System to the Formation of Amyloid, J. Exper. Med. **45**: 619, 1927.
21. Warren, S.: Generalized Amyloidosis of the Muscular Systems, Am. J. Path. **6**: 161, 1930.

OBSERVATIONS ON THE NORMAL EMPTYING TIME OF THE STOMACH OF THE DOG, USING A MIXED MEAL*

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IN 1933 Van Liere and Crisler[1] made a preliminary report on the normal emptying time of the stomach of the dog. During the past two or three years considerable more data has been obtained in the course of experimental work on various conditions influencing the emptying time of the stomach. We feel that these data, combined with those published in the preliminary report, will constitute fairly accurate figures, which may be of aid to other workers studying gastric emptying time, particularly in the dog.

Methods.—The standard meal used in all of these studies consisted of 40 gm. of hamburger, 10 gm. of dried ground bread, and 50 c.c. of milk. Fifteen grams of barium sulphate were added so that the gastric contents could be seen with the fluoroscope. The constituents of the meal were thoroughly mixed, and the animals were fed at the same time each morning. The animals were kept in a quiet room, free from extraneous influences, and were tethered to a chain about

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4 feet long so that the amount of exercise was limited. It must be pointed out here that the standard meal was not sufficient to keep a large dog well nourished, so from time to time substantial supplementary feedings were given. Care was taken, however, as to the time these feedings were given, as Ivy and Fauley[2] have shown that the state of hunger affects the gastric emptying time. Every effort was made to keep all experimental conditions as uniform as possible.

Results.—The accompanying table gives the results obtained on a series of 50 dogs. A total of 540 tests was made, including the original 200 tests published in the preliminary report.

	EMPTYING TIME OF THE STOMACH (IN HOURS)			
	AVERAGE	MEAN	LONGEST	SHORTEST
A 25 dogs	6.59	6.54	8.07	5.42
200 tests	6.61	6.62		
B 50 dogs	6.47	6.49	8.20	5.41
540 tests	6.54	6.52		

A = Those reported in the preliminary report.
B = Those previously reported plus 25 additional dogs.

Discussion.—It is distinctly interesting to note how remarkably well the new figures check with those given in the preliminary report. This very fact leads one to attach considerable significance to the values obtained. For example, the mean emptying time of the series of 25 dogs first reported was 6.54 hours, and the mean for the entire series was 6.49 hours, a difference of only 0.05 hours. It is also interesting to note that the extremes, too, seem to be well defined at about 5.41 and 8.20 hours, respectively.

We feel that the standard meal used has particular advantages, as it contained carbohydrate, protein, and fat. The milk gave it a desirable consistency, and the dogs ate it with relish, in spite of the fact that it contained considerable barium sulphate.

The dogs used were of various breeds and sizes so that the figures reported represent a good cross-section of what one might find in studying the gastric emptying time in the average laboratory animal.

Summary.—The emptying time of the stomach was determined in a series of 50 dogs. A total of 540 tests was made. The average emptying time of the stomach for the dogs was 6.47 hours and the average for the tests was 6.54 hours. The variations were between 5.41 and 8.20 hours, respectively. The emptying time of the stomach of each individual dog remained strikingly uniform from day to day. It is felt that these figures will be of interest and of aid to other workers.

REFERENCES

1. Van Liere, E. J., and Crisler, G.: Normal Emptying Time of Stomach of Dog, Proc. Soc. Exper. Biol. & Med. **31**: 85, 1933.
2. Ivy, A. C., and Fauley, G. B.: Effect of Hunger on Emptying Time of Stomach, Am. J. Physiol. **91**: 206, 1929.

BACTERICIDAL AND BACTERIOSTATIC PROPERTIES OF DIOTHANE SOLUTIONS*

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SINCE 1930, when the preparation of diothane was first described by Rider,[1] a number of papers have been published treating its chemistry, pharmacology, and clinical uses. Diothane, a local anesthetic, is the hydrochloride of piperidinopropanediol di-phenylurethane.

For clinical purposes, diothane is usually employed as a 1 per cent solution, which represents near saturation. Because of its tendency to precipitate from its solutions as the free base, piperidinopropanediol di-phenylurethane, it is necessary that considerable care be given to its preparation, particularly with respect to adjustment of pH. The solution must be stored in special non-alkaline glass containers, preferably of Pyrex, or of similar brand.

Due to the fact that diothane solution is frequently administered by injection, the question of proper means for its sterilization is important. Adequate sterilization may be achieved by boiling, since it has been shown that heating to boiling temperature for moderately prolonged periods of time did not alter its activity as determined by physiologic tests.[2] The degree of decomposition resulting after boiling for periods as long as eighteen hours, as measured by its aniline content,[3] was very slight.

As a result of carrying out sterility tests on diothane solution, sterilized by various means, it was soon learned that in 1 per cent concentration it possessed considerable bactericidal activity and that weaker solutions were very strongly bacteriostatic. Further work indicated that this activity was even more pronounced than first described in earlier publications.[4, 5]

When the preliminary work on this phase of the problem was carried out, it was noted that considerable clouding occurred whenever even small amounts of broth culture were added to diothane solutions. It was learned that this was due to the precipitation of the free base as a result of a change in the pH following the addition of the more alkaline broth culture. In preceding papers, it was reported that diothane solutions possessed comparatively low killing powers against certain organisms, notably *Staph. aureus*. It is now known that this was due, in part at least, to partial inactivation of the diothane solution by the addition of alkaline broth cultures.

Attempts to buffer the various media used in growing the test organisms, or to adjust such cultures after growth took place, proved unsuccessful. In order to prevent subsequent clouding by this means, it was necessary to adjust the pH to a reaction which proved to be incompatible with the growth of the organ-

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isms. This difficulty was overcome, however, by the use of washed organisms suspended in physiologic saline solution.

Cultures were grown either in liquid, or on solid media for the usual period of time. The organisms were then removed from the media by washing with sterile physiologic saline solution, in the case of the solid media, and centrifuged, or by centrifugation if liquid media were employed. They were then resuspended in sterile physiologic saline solution, aseptic technic being observed in all operations. Unless otherwise indicated, this technic was employed throughout the tests described below.

BACTERICIDAL ACTIVITY OF DIOTHANE SOLUTIONS

To 5 c.c. quantities of diothane solution of 1 per cent concentration was added such an amount of suspended organisms, treated as described above, as would be equivalent to 0.5 c.c. of a twenty-four-hour broth culture. After various intervals indicated in the table, transfers were made to F.D.A. formula nutrient broth. Readings were made after seven days' incubation at 37.5° C.

TABLE I

BACTERICIDAL ACTIVITY OF DIOTHANE SOLUTION

ORGANISM	TIME OF EXPOSURE IN MINUTES						
	5	10	15	30	60	120	240
B. coli	–	–	–	–	–	–	–
B. typhosus	–	–	–	–	–	–	–
Sarcina lutea	–	–	–	–	–	–	–
Staph. aureus	+	+	+	–	–	–	–
	TIME OF EXPOSURE IN HOURS						
	1	2	4	8	24	48	96
B. subtilis	+	+	+	+	+	+	+

It will be noted that with the exception of *Staph. aureus* and *B. subtilis,* all of the organisms, comprising a representative group such as is usually recommended for testing the efficacy of bactericidal substances, were killed within five minutes or less. In the case of *Staph. aureus,* killing was accomplished only after exposure for thirty minutes or longer. In control tests, it had been shown previously that the strain of *Staph. aureus* used was such as to resist the action of a 1:60 phenol solution for a period of five minutes.

Preliminary tests indicated that diothane solution killed *B. subtilis,* however, microscopic examination of the cultures employed proved them to be free of spores. When older cultures were used killing was not accomplished even after exposure of four days or longer to 1 per cent diothane solution.

Assuming that there might be a difference in the killing power of diothane solution against spores in the wet state as compared with similar cultures which were dried, small amounts of washed *B. subtilis* organisms were soaked into small sterile squares of filter paper. These were then dried at incubator temperature for ninety-six hours in sterile Petri dishes. Several pieces of the paper, containing the organisms in dried form, were introduced into the 1 per cent

diothane solution. After thoroughly agitating the paper in this solution, transfers were made to sterile broth. In all cases the organisms treated in this manner survived exposure equally well for periods of four days, or even longer. The culture of *B. subtilis* used in the tests described in Table I contained numerous spores.

FUNGICIDAL ACTIVITY OF DIOTHANE SOLUTIONS

Information regarding the killing power of diothane solutions against molds and yeasts is desirable. Accordingly, its action was tested against a culture of yeast, two strains of Penicillium and one of Aspergillus. Each of these organisms had been isolated from the laboratory and were frequently found as contaminants in other materials.

Suitable amounts of the cultures were inoculated in 5 c.c. quantities of 1 per cent diothane solution and after the intervals noted, transfers were made into sterile broth. For the molds, Sabouraud's medium was employed, and for the yeast, dextrose broth was used. Readings were made after one week incubation at 25 to 30° C.

TABLE II

FUNGICIDAL ACTIVITY OF DIOTHANE SOLUTION

ORGANISM	TIME OF EXPOSURE IN HOURS						
	1	2	3	4	8	24	48
Penicillium "A"	−	−	−	−	−	−	−
Penicillium "B"	+	+	−	−	−	−	−
Yeast	−	−	−	−	−	−	−
Aspergillus niger	+	+	+	+	−	−	−

It was found that each of the strains of yeast and molds was killed after eight hours' exposure to a 1 per cent diothane solution, the yeast being more readily killed than the others. Previous microscopic examination showed that the mold cultures contained numerous spores.

BACTERIOSTATIC PROPERTIES OF DIOTHANE SOLUTION

In spite of the possession of a rather limited bactericidal activity, it was found that diothane solutions are strongly bacteriostatic. In the tests to be described a larger number of organisms was employed, including in the group several pathogens. In order to learn something of the possible difference in activity at different pH values, each lot of media was divided in two parts, one of which was adjusted to pH of 6.8 and the other to pH of 7.8. As might be anticipated, the greater amount of precipitation occurred in those lots of media having the more alkaline reaction.

Beginning with an aqueous solution of diothane of a concentration of 1:200 or 0.5 per cent, various dilutions were prepared by adding calculated amounts to sterile media. Inoculations were made directly into the broths thus prepared. The tubes were examined for growth after seven days' incubation. From those tubes showing no growth, transfers were made into sterile broth in order to determine whether lack of bacterial development in the first tubes indi-

cated lethal, or merely inhibitory action. The following Tables III and IV present the results in condensed form.

In the tables, the minus sign (–) indicates death of the organisms, the plus sign (+) indicates growth, while the letter "i" indicates that the organisms are inhibited only. It will be noted that the diothane solution is most active in the acid media and in all instances was less active in those media possessing the more alkaline reaction. It will also be seen that in the acid media all of the organisms with the exception of *B. subtilis* were killed when such media contained as low a concentration of diothane as 1 part in 400 parts of broth. *C. diphtheriae*, *B. typhosus*, and *Sarcina lutea* were readily killed, while *B. subtilis* was not

TABLE III

BACTERIOSTATIC ACTIVITY OF DIOTHANE IN BROTH OF pH 6.8

CONCENTRATION OF DIOTHANE IN BROTH OF ORIGINAL pH OF 6.8	pH	B. TYPHOSUS	B. COLI	STAPH. AUREUS	B. SUBTILIS	S. LUTEA	C. DIPHTHERIAE	YEAST	PENICILLIUM
1: 200	5.6	–	–	–	i	–	–	–	–
1: 400	5.9	–	–	–	i	–	–	–	–
1: 800	6.4	–	+	i	i	–	–	–	–
1: 1,600	6.4	–	+	i	i	–	–	–	+
1: 3,200	6.4	–	+	+	i	–	–	+	+
1: 6,400	6.5	+	+	+	+	–	–	+	+
1:12,800	6.8	+	+	+	+	+	+	+	+

TABLE IV

BACTERIOSTATIC ACTIVITY OF DIOTHANE IN BROTH OF pH 7.8

CONCENTRATION OF DIOTHANE IN BROTH OF ORIGINAL pH OF 7.8	pH	B. TYPHOSUS	B. COLI	STAPH. AUREUS	B. SUBTILIS	S. LUTEA	C. DIPHTHERIAE	YEAST	PENICILLIUM
1: 200	5.6	–	–	–	i	–	–	–	–
1: 400	6.6	–	+	–	+	–	–	–	–
1: 800	7.4	+	+	+	+	–	–	–	–
1: 1,600	7.6	+	+	+	+	–	–	+	+
1: 3,200	7.6	+	+	+	+	+	+	+	+
1: 6,400	7.8	+	+	+	+	+	+	+	+
1:12,800	7.8	+	+	+	+	+	+	+	+

killed in broth containing diothane to the extent of 1:200. It was significant that the growth of *B. subtilis* was readily inhibited in broth containing as little as 1 part of diothane to 3,200 parts of broth. It was found further that broth containing varying amounts of diothane is lethal and strongly inhibitory to the strains of yeast and molds employed in the tests.

The change in reaction in the media following the addition of diothane is noted by the pH values recorded in the second column.

CONCLUSIONS

It must be concluded that while a 1 per cent diothane solution is rather strongly bactericidal against most of the organisms usually employed to measure such activity, since it does not kill *B. subtilis* spores even after contracted periods of exposure, it may not be considered to be "self-sterilizing." How-

ever, from a consideration of the foregoing results it is at once apparent that a 1 per cent solution of diothane, once sterilized by boiling, is well preserved and will remain sterile if ordinary aseptic technic is observed during the removal of small amounts of the solution from its container.

It is reasonable to assume that such bacteria, with the exception of spore bearers, as may become accidentally introduced into the solution will be killed within an hour or so. The results with the molds indicate that a longer period of exposure will be required to kill them.

SUMMARY

1. Diothane solution in concentration of 1 per cent is strongly bactericidal against the more common contaminants with the exception of *B. subtilis* and probably other spore formers.

2. In concentration of 1 per cent, diothane solution will kill a resistant strain of *Staph. aureus* within one hour, and *B. typhosus* and *B. coli* within five minutes.

3. The spores of *B. subtilis* will resist the activity of 1 per cent diothane solution for four days or longer.

4. *B. subtilis* is inhibited in broth containing diothane in concentration as low as 1:3200.

5. Molds and yeasts are killed by diothane solutions.

6. While strongly bactericidal against many organisms, diothane solution may not be considered to be "self-sterilizing" because of its weak action against *B. subtilis* spores.

REFERENCES

1. Rider, T. H., and Hill, A. J.: Studies of Glycidol. I. Preparation From Glycerol Monochlorohydrin, J. Am. Chem. Soc. **52**: 1521, 1930.
2. Rider, T. H.: Piperidinopropanediol Di-phenylurethane Hydrochloride, a New Local Anesthetic, J. Pharmacol. & Exper. Therap. **47**: 255, 1933.
3. Cook, E. S., Bambach, K., and Rider, T. H.: The Stability of Diothane Solution. II, J. Am. Pharm. A. **24**: 269, 1935.
4. Rider, T. H.: Pharmacological and Preliminary Clinical Observations on Diothane, a New Local Anesthetic, Anesth. & Analg. **12**: 259, 1933.
5. McKim, G. F., Smith, Parke G., Rush, T. W., and Rider, T. H.: Use of Diothane as a Local Anesthetic in Urology, J. Urol. **29**: 277, 1933.

THE POSSIBLE IMPORTANCE OF MEDIUMS CAUSING SURFACE AND SUBSURFACE GROWTH OF PATHOGENIC FUNGI TO THE DIAGNOSIS AND TREATMENT OF DISEASE*

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IN MY work I have found that solid mediums may act consistently in two ways with reference to growth of pathogenic fungi. They may produce a preponderance of either surface or subsurface growth.[1, 2] Workers are familiar with the fact that there is a variable amount of subsurface growth in the case of certain organisms. In mediums of pH 5.6 containing 1 per cent cysteine-cystine as the source of nitrogen and 4 per cent dextrose as the source of sugar, growth is predominantly subsurface, while in similar mediums with peptone substituted for cysteine-cystine, it is predominantly surface. About a month after good subsurface growth had been obtained on cysteine-cystine, in most instances by pouring over the surface a thin layer of Sabouraud's medium, previously subsurface organisms grew on the surface.

The following pathogenic fungi and two nonpathogenic saprophytes, *Lichthemia sp.* and *Scopulariopsis brevicaulis*, were studied: *Achorion schoenleinii, Acladium castellani, Candida candida, Endodermophyton indicum, Endodermophyton dermatitidus, Epidermophyton cruris, Epidermophyton inguinale, Epidermophyton rubrum, Glenospora Gammeli, Geotrichum Bachmann, Indiella americana, Microsporon Audouini, Microsporon felineum, Microsporon gypseum, Monosporium apiospermum, Monilia albicans, Oospora humi, Sporotrichum Schenckii, Trichophyton balcaneum, Trichophyton crateriforme, Trichophyton decalvans, Trichophyton granulosum, Trichophyton gypseum, Trichophyton gypseum-asteroides, Trichophyton gypseum-lacticolor, Trichophyton interdigitale, Trichophyton japonicum, Trichophyton louisianicum, Trichophyton niveum, Trichophyton purpureum, Trichophyton sulfureum, Willia anomala.* In order to obtain subsurface growth these organisms were incubated for forty days on mediums consisting either of hydrolyzed hair (Difco Laboratories prepared it for this work) 1 per cent or cysteine hydrochloride Eastman 1 per cent, dextrose 4 per cent, agar 1½ per cent (pH 5.6). The growths were then overlaid with several millimeters of Sabouraud's proof medium and reincubated for forty days. The incubations took place at room temperature in diffused light.

Of the 34 organisms, Sabouraud's medium failed to bring four to the surface: *Endodermophyton tropicale, Candida candida, Trichophyton balcaneum,* and *Willia anomala.* It is possible that in these instances the organisms were dead or that the medium was too hot when poured over them. In some instances

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the growths seemed almost as profuse as on Sabouraud's proof medium. This, however, is not to be expected since the layer of Sabouraud's is only a few millimeters deep.

The ability of the Sabouraud's proof medium to attract organisms from the subsurface suggests its potential use in packs to be placed on areas of chronic disease with several objects in view. First, it might serve to attract the etiologic agent to the surface. Second, if an antiseptic were incorporated in it, it might not only attract to the surface but kill. Third, it might attract organisms to the surface so that in areas from which cultures have been previously negative, diagnosis might be made. When made, the applications should be kept moist either by covering them with adhesive or other means. Either the liquid medium can be used, or the solid medium incorporated in small gauze sponges. The mediums should be sterilized.

It is possible that other mediums than Sabouraud's might be more proficient. Sabouraud's in my experience, however, produces as aerial type of growth as any.

The difficulty with reference to antiseptics where chronic lesions are below the superficial layers is apparent. Any method which would efficiently dislodge organisms located deep might prove effective by allowing lethal antiseptics to contact them.

The cysteine-cystine mediums allow subsurface growth to a depth of usually not more than 1 cm.[1, 2] The depth with reference to proportional concentrations of cysteine-cystine in the skin may help explain chronicity in skin diseases. With reference to bacteria our results have not been enlightening.

REFERENCES

1. Williams, J. W.: Invasiveness of Skin Infections Caused by Pathogenic Fungi and Subsurface Mycelium, Science **83**: 396, 1936.
2. Idem: Subsurface Growth of Pathogenic Fungi on Hair, Pig Skin and Cysteine-Cystine Mediums, Arch. Dermat. & Syph. In press.

THE QUANTITY OF IODINE IN THE THYROID GLAND OF THE RABBIT AND THE INFLUENCE THEREON OF A DIET OF CABBAGE*

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THE purpose of the investigation reported here was to establish the amount of iodine in the thyroid gland of the normal rabbit and the proportion of iodine to the weight of the gland, and to compare with this the iodine content of the thyroid glands of rabbits fed for varying lengths of time on a diet consisting almost exclusively of cabbage. At the same time the effect of the cabbage diet on the physical condition as evidenced by body weight was noted.

Various investigators have reported the development of simple goiter in the rabbit apparently as the result of a cabbage diet. Webster[1] recorded some very striking results from which the conclusion is drawn that cabbage grown in certain localities and at certain seasons, particularly during the winter, contains a goitrogenic agent that is much more powerful to produce goiter than is a diet that is deficient in iodine only. The suggestion is made that the cabbage depletes the iodine store of the thyroid gland and that this may be due to a reducing agent acting on the oxidation-reduction systems of the body and causing an increased demand for thyroxin to activate oxidation. McCarrison[2] reported the incidence of goiter as 38.9 per cent in rabbits fed on a mixture of winter cabbage 60 parts, sorghum 20 parts, and whole wheat 20 parts. In rabbits subjected to insanitary conditions and the same imperfect diet as before, he states that goiter developed in 88.9 per cent. As the Edmonton district of western Canada has long been regarded as an area in which goiter, particularly of the myxedematous type, is prevalent, and as cabbage is a much used food, it seemed desirable to study the question of a possible relation between cabbage in the diet and the occurrence of goiter.

For our experiment the diet employed by Webster in the article previously quoted was adopted. This provides for each rabbit: cabbage 250 gm. daily, oats 50 gm. weekly, and hay 20 gm. weekly. Control rabbits were fed on full rations of oats, turnips, carrots and hay. Cabbage was excluded from the food supplied to the control animals. All the food used was grown locally. The animals were caged in pairs and the environmental conditions were the same for all. The total number of animals used was 81, of which 48 were given the cabbage diet and 33 were used as normal controls. The length of time the experiment lasted in individual cases varied from four weeks to twelve. Most of the determinations were made after four-, eight-, and twelve-week periods with a few at the intervening weekly intervals. The procedure was to weigh the animal, to examine it for any evidence of goiter by palpation, and to start it on either the cabbage or the control diet. At the expiration of the predetermined number of

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weeks the animal was weighed, killed rapidly by the inhalation of chloroform vapor, and the thyroid removed. The gland was dried between filter papers for five minutes and weighed. It was then dried at a temperature of 100° C. for forty-eight hours and reweighed. Subsequently all the glands were dried again in the oven as before, weighed, and the iodine content of each was determined.

It might be noted that as a preliminary measure tests were made as to the best way to kill the rabbits. It was found in every case where the neck blow was employed that all the tissues of the neck, including the thyroid gland, were thereby rendered congested. The gland was usually found engorged with blood when death was produced by this means, but this was not the case when chloroform was used.

RESULTS

The control animals showed a gain in weight up to eight weeks, but those kept for twelve weeks showed an insignificant loss in weight. The cabbage rabbits, on the contrary, showed marked loss of weight (Table I). All the rabbits used were young adult animals and litters were divided, so far as possible, between the two main groups. In view of the other findings, this loss of weight is to be regarded simply as evidence of inanition.

TABLE I

	CONTROLS AVERAGE BODY WEIGHT				CABBAGE AVERAGE BODY WEIGHT		
	GRAMS		GAIN OR LOSS		GRAMS		GAIN OR LOSS
	BEGIN	END	%		BEGIN	END	%
4 wk.	2010.0	2260.0	+12.4	4 wk.	2057.0	2271.0	+10.4
8 wk.	1612.0	2033.0	+26.1	8 wk.	2122.0	2003.0	- 5.6
12 wk.	2461.0	2417.0	- 1.8	12 wk.	2398.0	1806.0	-24.7

Little importance can be attached to the weight of the fresh thyroid. The amount of water in the gland is very variable. This is exemplified by our records of the individual glands which show a loss of weight when dried from a minimum of 50.2 per cent to a maximum of 80.9 per cent in the control group and from 57.4 per cent to 84.3 per cent in the cabbage group. When the weight of the thyroid gland that has been dried is compared with body weight, it is found to constitute from 0.0038 per cent to 0.0065 per cent in all groups, cabbage and control, except the one comprising animals maintained on cabbage for four weeks. In this group the average weight of the dried thyroid and its proportion to body weight are much greater than in any other group, cabbage or control. This is due almost entirely to one gland, which had a weight when dried of 1,504.9 mg.

The method employed to determine the iodine content of the thyroid glands analyzed was described by Remington, McClendon, von Kolnitz and Culp.[3] In connection with these analyses the following comment should be made. A source of error in the colorimetric determination of iodine is the variation of the ratio of iodine in the carbon tetrachloride to the iodine in the water. The amount of iodine remaining in solution in the water varies with the concentration of salts in the water. In these analyses the concentration of salts varied with each run and, since the effect of the variation was not determined, an error was introduced.

The amount of iodine in the thyroid gland of the normal rabbit averaged 0.033 mg. The maximum quantity in one gland was 0.071 mg. and the minimum 0.013 mg. In the case of the rabbits fed on cabbage, the highest average iodine content, 0.062 mg., occurred in those that had been given the restricted diet for four weeks. The maximum in this group was 0.075 mg. and the minimum 0.037 mg. The rabbits maintained on cabbage for eight weeks showed an average iodine content of the gland of 0.038 mg., with a range from 0.041 mg. to 0.030 mg. Where the cabbage diet was continued for twelve weeks, the amount of iodine in the thyroid averaged 0.031 mg., with 0.062 mg. and 0.011 mg. at the two extremes. It will be observed that the range is almost the same in the two groups, control and cabbage. The maximum quantity found in one gland is almost the same in the two groups and the minimum is likewise practically the same.

The rabbits that had been on cabbage for four weeks yielded the highest average weight for the dried thyroid, the highest average proportion of weight of dried gland to body weight and the highest average total iodine. The average iodine content of the glands of these rabbits is nearly double that of any other group, but when expressed as a percentage of the weight of the gland, it is the lowest of all. It is possible that this is evidence of the existence of goiter and even of a goiter-producing action on the part of cabbage. When it is observed, however, that rabbits subjected to a diet of cabbage for longer periods show thyroid glands that in size, proportion to body weight, and iodine content are practically identical with those of animals maintained on a full diet, it seems necessary to conclude that, even if the cabbage was a causative agent in the departure from normal observed, the disturbance was a temporary one.

TABLE II

	AVERAGE BODY WEIGHT AT DEATH GRAMS	AVERAGE WEIGHT OF THYROID ON REMOVAL MILLIGRAMS	PROPORTION OF THYROID TO BODY WEIGHT %	AVERAGE WEIGHT OF THYROID DRIED MILLIGRAMS	PROPORTION OF DRIED THYROID TO BODY WEIGHT %	AVERAGE TOTAL IODINE MILLIGRAMS	PROPORTION OF I TO WEIGHT OF DRIED THYROID %
				Controls			
4 wk.	2260.0	622.5	0.028	100.2	0.0044	0.033	0.033
8 wk.	2033.0	773.0	0.038	113.7	0.0056	0.032	0.028
12 wk.	2417.0	300.0	0.012	92.5	0.0038	0.035	0.038
				Cabbage			
4 wk.	2271.0	1785.3	0.079	512.0	0.0226	0.062	0.012
8 wk.	2003.0	1435.7	0.072	106.9	0.0053	0.038	0.036
12 wk.	1806.0	569.3	0.032	117.7	0.0065	0.031	0.026

SUMMARY

1. The average amount of iodine in the thyroid gland of the normal rabbits studied was 0.033 mg., the average weight of the dried gland was 101.6 mg., and the iodine content equalled 0.033 per cent of the gland by weight.

2. The average amount of iodine in the thyroid gland of rabbits kept on a diet of cabbage for four weeks was 0.062 mg., the average weight of the dried gland was 512.0 mg., and the iodine content equalled 0.012 per cent of the gland by weight.

3. Rabbits maintained on a diet of cabbage for periods of eight and twelve weeks had thyroid glands that in size and amount of iodine contained were the same as those of the control animals.

4. Rabbits kept on a diet consisting almost exclusively of cabbage invariably lost weight.

The iodine determinations reported in this paper were made by Mr. T. L. Cairns, whose assistance is gratefully acknowledged.

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REFERENCES

1. Webster, B.: Studies in the Etiology and Nature of Simple Goiter as Produced Experimentally in Rabbits, Trans. Am. Assn. Study Goiter, p. 12, 1931.
2. McCarrison, R.: Food and Goitre, Brit. M. J. 2: 671, 1933.
3. Remington, R. E., McClendon, J. F., von Kolnitz, H., and Culp, F. B.: The Determination of Traces of Iodine. IV. Iodine in Small Quantities of Thyroid and Other Tissues, J. Am. Chem. Soc. 52: 980, 1930.

THE POLLEN CONTENT OF THE AIR OF SAN DIEGO, CALIFORNIA*

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WITH THE TECHNICAL ASSISTANCE OF MRS. HELEN MCMICHAEL

A KNOWLEDGE of the pollen content of the air of a given locality is essential for the intelligent study and treatment of allergic diseases associated with pollen sensitization in that locality. This is particularly true of Southern California because of the diversity of vegetation and the influence upon it of the climatic conditions peculiar to that part of the country.

In order that we might have a basis upon which to work in the treatment of these conditions in San Diego, a study of the pollen content of the air of that city, extending over a period of two and one-half years, was made. Slides for the collection of pollen grains were exposed each day at the Clinic station during this time. From May 1, 1934, to Nov. 1, 1934, pollens were collected at three additional stations in the city, these stations representing, as far as possible, the varying topographical characteristics of the different sections of the city. At each station, four slides were placed in upright position, each slide facing one of the four points of the compass so that pollen was collected from all directions. A shelter to protect the slides from rain but open on the four sides was provided. Each set of slides was exposed for twenty-four hours.

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The number of pollen grains collected during this interval was determined by counting the grains contained in an area one inch square on the slide. Daily logs of the pollens collected at the different stations were kept and charts of the findings made.

Chart 1.

Chart 1 is a relief map of the city of San Diego and its immediate environs. The locations of the four pollen collecting stations are indicated on the map.

Chart 2 compares the total pollen counts from May to November, 1934, at the four stations. At all stations the slide facing west contained the greatest number of pollen grains with rare exceptions; the slides facing south, north, and east contained the next largest numbers in the order named except that during

the winter months there were frequently more pollen grains on the slide facing north than on the one facing south. As might be expected, the pollen content of the air was lowest at Sunset Cliffs which is situated on the ocean. It is interesting to note that the count was greatest at the stations located in the central (Clinic) and southern (Logan Heights) portions of the city, even though the western (State College) station is considerably farther inland.

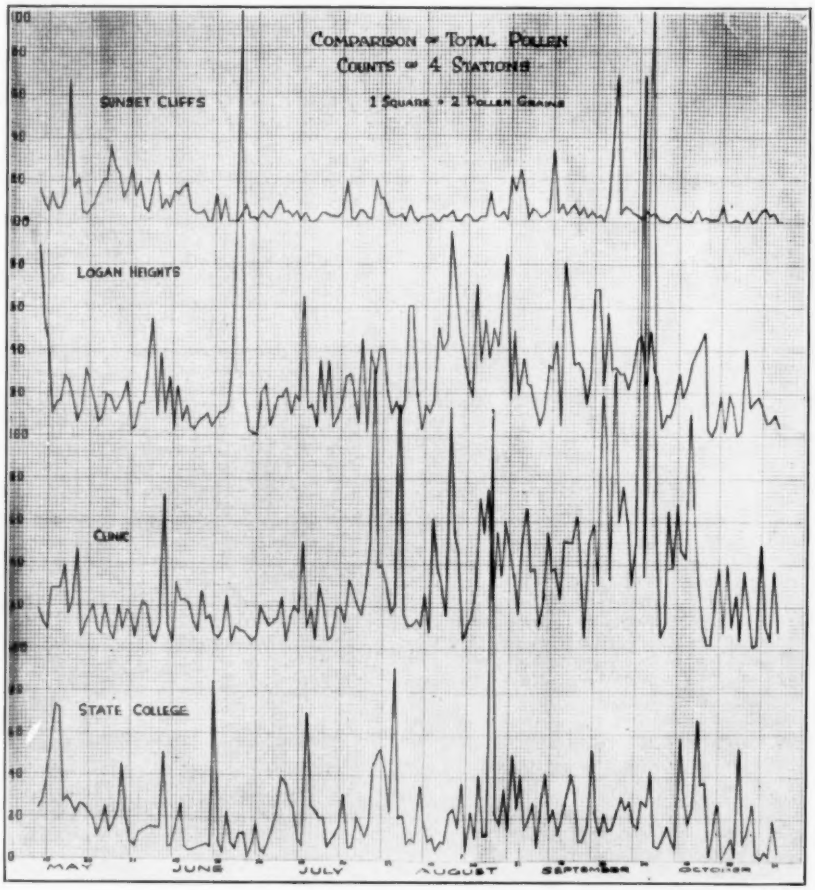

Chart 2.

Charts 3 and 4 show the daily total pollen counts at the Clinic for the morphologically distinct groups of pollens, and the daily hourly wind velocity, rainfall, noon temperature, percentage of sunshine and relative humidity for the city for the years 1933, 1934, and 1935 (to May, 1935).

Chart 5 gives the pollen counts of the cypress, oak, cottonwood, and olive trees for the years 1933, 1934, and 1935 (to May, 1935) at the Clinic station.

Knowledge of certain meteorologic data concerning San Diego is essential to the intelligent understanding of these charts. The daily variations are recorded graphically. They may be summarized and amplified as follows:

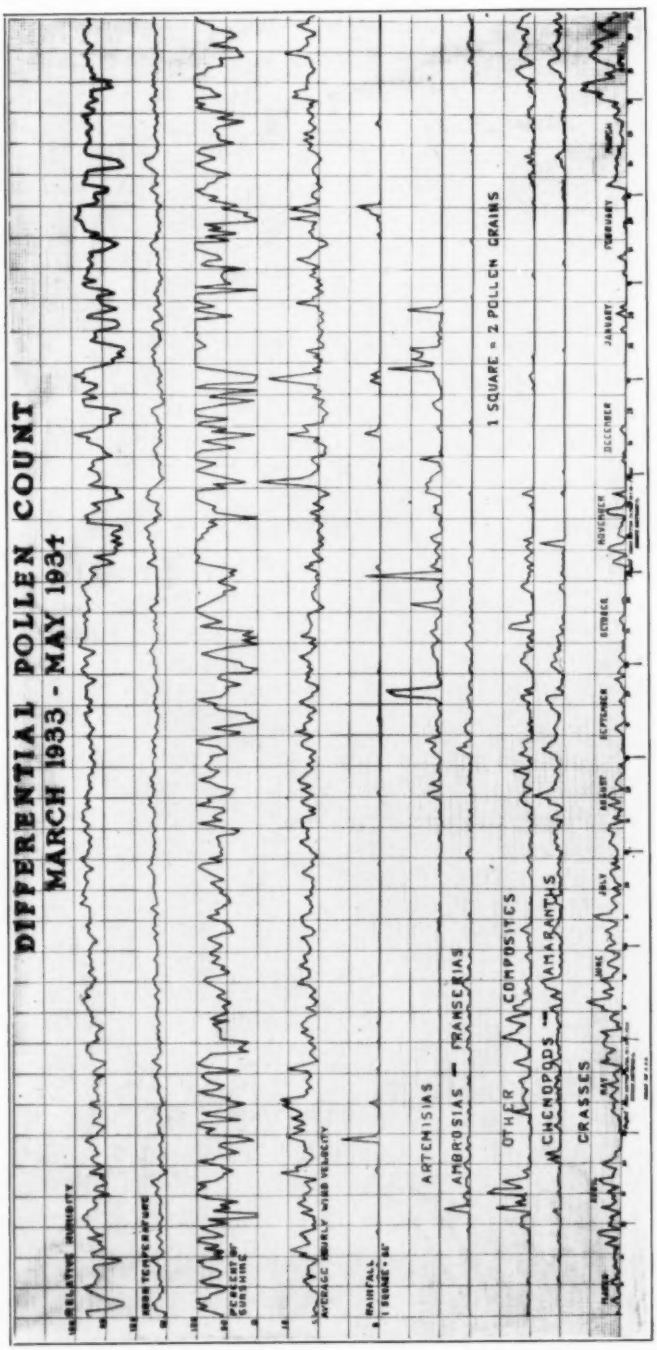
DIFFERENTIAL POLLEN COUNT
MARCH 1933 - MAY 1934
RELATIVE HUMIDITY
HOURLY TEMPERATURE
PERCENT SUNSHINE
AVERAGE HOURLY WIND VELOCITY
RAINFALL
1 SQUARE = 2 POLLEN GRAINS
ARTEMISIAS
AMBROSIAS — FRANSERIAS
OTHER COMPOSITES
CHENOPODS — AMARANTHS
GRASSES
MAY
JULY
AUGUST
SEPTEMBER
OCTOBER
NOVEMBER
DECEMBER
JANUARY
FEBRUARY

Chart 3.

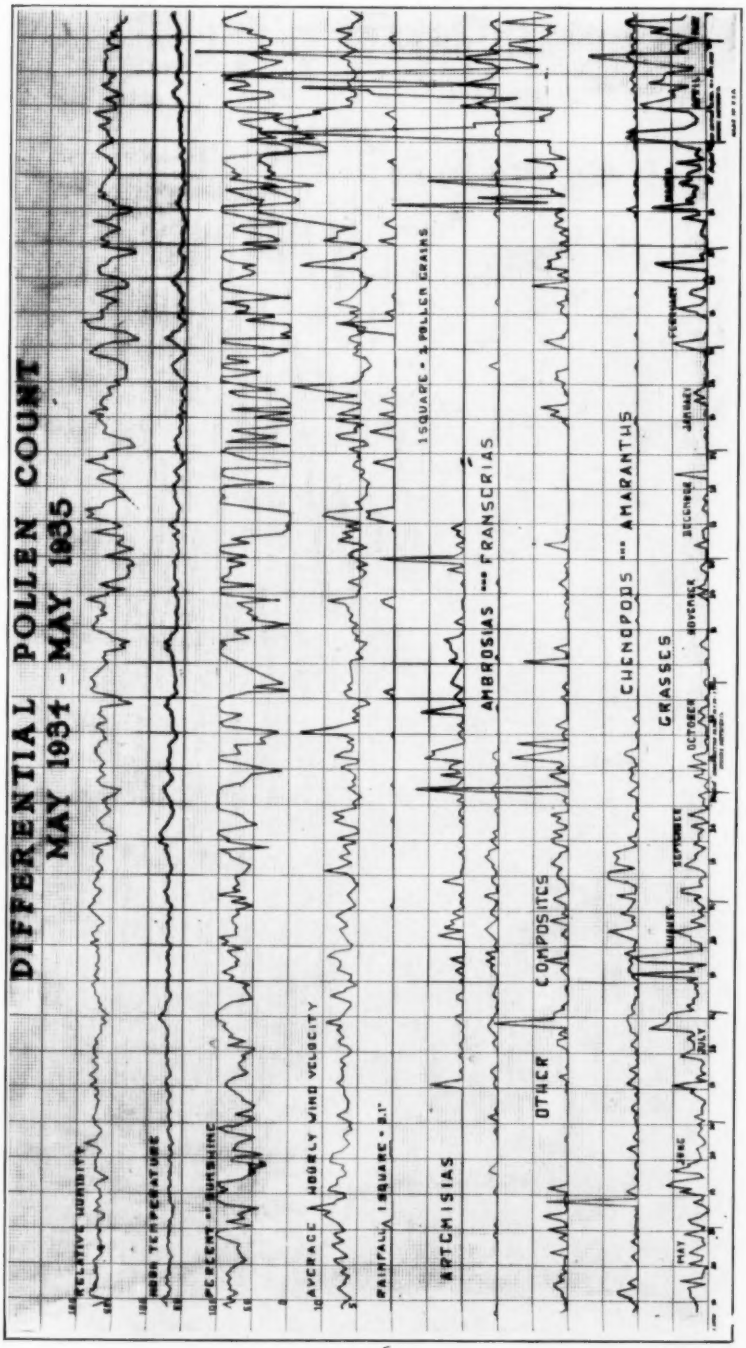
DIFFERENTIAL POLLEN COUNT
MAY 1934 - MAY 1935
RELATIVE HUMIDITY
HOURLY TEMPERATURE
PERCENT OF SUNSHINE
AVERAGE HOURLY WIND VELOCITY
RAINFALL 1 SQUARE = .01"
1 SQUARE = 1 POLLEN GRAINS
ARTEMISIAS
AMBROSIAS --- FRANSCRIAS
OTHER COMPOSITES
CHENOPODS --- AMARANTUS
GRASSES
MAY
JUNE
JULY
AUGUST
SEPTEMBER
OCTOBER
NOVEMBER
DECEMBER
JANUARY
FEBRUARY

Chart 4.

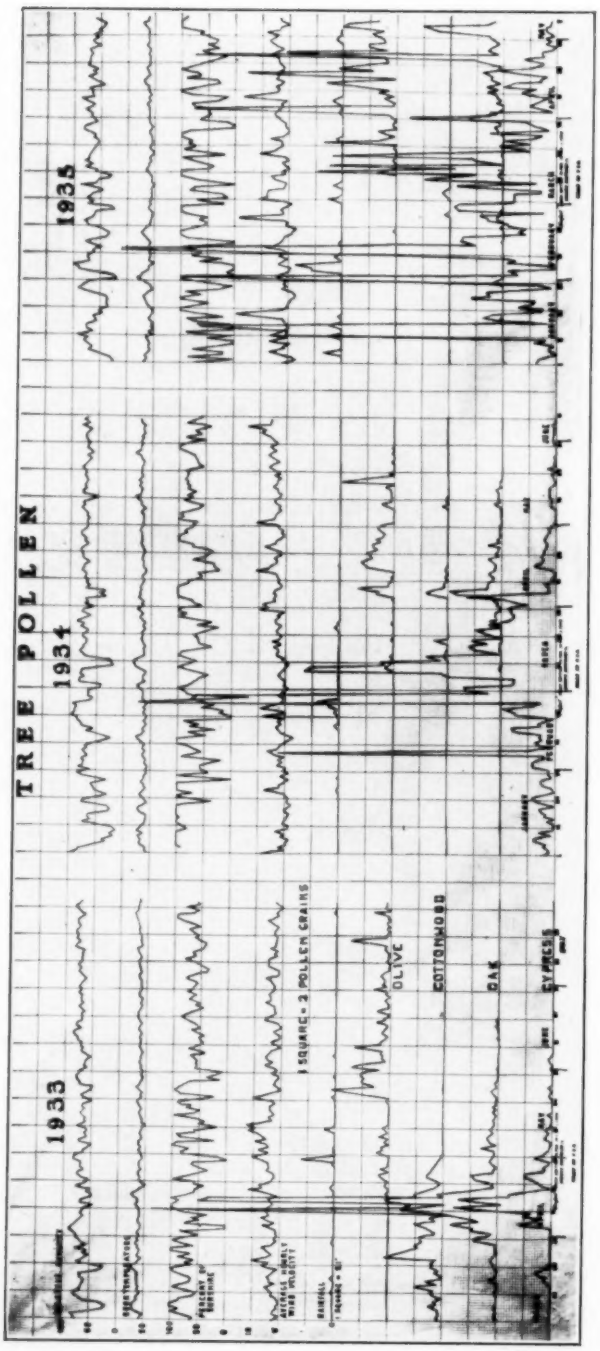
TREE POLLEN
1933
1934
1935
1 SQUARE = 2 POLLEN GRAINS
OLIVE
COTTONWOOD
OAK
CYPRESS

Chart 5.

1. The mean annual precipitation is about 10 inches, the rains being confined almost exclusively to the winter months. Marked variations in amount from year to year are common.

2. The prevailing direction of the wind is from the ocean about 90 per cent of the time. The deviations occur almost entirely during the winter season.

3. The hourly wind velocity is rarely less than 5 or more than 15 miles per hour.

4. The mean annual temperature variation is approximately 13° F. However, diurnal variations of as much as 30° F. may occur. Killing frosts are unknown.

5. The relative humidity averages 75 per cent.

Undoubtedly the most important factor in determining the pollen crop in a locality for a given year is the amount of rainfall during the growing season. This is shown by the corresponding differences in rainfall and in pollen crops for the years 1933-1934 and 1934-1935. The amount of rainfall in the winter of 1933 and 1934 was about 5 inches; in the winter of 1934 and 1935 it was about 15 inches. The total pollen crop in the first half of 1935 was approximately double that of the corresponding period for 1934. The pollen concentration of the air during the long arid summer is maintained to a certain extent by the pollen from some of the anemophilous plants which depend largely upon dew or irrigation for moisture.

Owing to the equable climatic factors, the pollinating seasons of the various species are less sharply demarcated here than in localities in which marked seasonal variations in climate occur. Moreover, the pollinating dates of the same plant may vary widely in different years. For example, the pollinating season for the cedar started in March in 1933 and in January in 1934 (Chart 5). Furthermore, pollens of various species appear on the slides long after the known pollinating periods of those species have ended. This is undoubtedly explained by the fact that, due to the absence of rainfall during the summer, pollen is not washed out of the air. Also, that which falls on the leaves of the plants may remain there until it is dislodged by a gust of wind, thus disseminating more pollen into the air.

The amount of pollen from a given plant may vary markedly from year to year. This point is illustrated by the fact that the pollen from the oaks was very plentiful in 1933 and relatively small in amount in 1934 and 1935. The pollen count of the cypress on the other hand was low in 1933, high in 1934 and very high in 1935 (Chart 5).

If the actual pollen concentration of the air in this city were compared with the extremely high values which obtain in any midwestern city, it might be assumed that pollinosis is a negligible problem here. This is far from the truth as many sufferers who have lived in both localities will attest. The lack in pollen concentration is made up to a large extent by the prolongation of the season and the diversity of vegetation. Allergic disease associated with pollen sensitization is a perennial disorder in San Diego.

I wish to express my appreciation to Dr. George F. Harsh, recently with the Allergy Clinic of the University of Chicago, and now associated with this Clinic, for his helpful criticism of this paper.

VARIATIONS IN THE BLOOD CHOLESTEROL OF MAN OVER A TIME PERIOD*

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IT HAS been pointed out from time to time that single duplicate estimations of any chemical compound or element in the body fluids are not representative; that under various conditions, artifically or naturally induced, the chemical equilibrium of the body alters itself, this state of affairs occurring even under basal conditions.

Thus, the lipids in the blood of man and animals have, in general, been considered to be variable, such variability being strongly altered by the ingestion of food. These statements have their origin in work done years ago, for instance, by Ahlfeld[1] who noticed that the serum of an animal becomes definitely milky after a meal rich in fat. In more recent years, while it has been shown that such a lipemia occurs in carnivorous animals after intensely fat meals,[2-5, 14] it has not been demonstrated to be true for herbivorous animals, and practically not at all for man.[6-11]

Man and Gildea,[12] evidently feeling that in these experiments the relationship between the weight of the individual and the weight of the fat administered had been neglected, noted that an alimentary lipemia could be produced in man, provided 3.5 gm. of fat per kilo of body weight be given. They also found that a smaller ration containing about 0.5 gm. of fat per kilo resulted in variations which were unreliable. Boyd[13] pointed out that these experiments indicated that well over 200 gm. of fat must be taken by a man at one meal in order that an alimentary lipemia might be produced; and since a normal person under normal conditions and doing a moderate amount of muscular work consumes much less than 200 gm. of fat at one meal, or at three meals for that matter, it was highly improbable that in such a man an alimentary lipemia ever occurred under normal conditions. Then Boyd proceeded one step further and in attempting to discover whether or not there was any variation in the concentration of blood lipids in man during a twenty-four-hour period, found but slight variations. The deviation per person per day for free cholesterol was 6.5 per cent and for ester cholesterol, 7.2 per cent. The variation in blood cholesterol from one person to another per twenty-four-hour period he found to be two to three times as great as the average variation per person per day. Burger and Somach[6] obtained similar results for total cholesterol in the whole blood of man during a similar period.

Thus, for a man consuming three ordinary meals per day, there is no existing evidence that any marked alteration in the blood cholesterol would be found for any given twenty-four-hour period. It is the purpose of this paper to extend

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this knowledge one step further; i.e., to determine the variation of blood cholesterol in man consuming three ordinary meals per day over a series of weekly periods. The importance of clarifying this aspect of the problem of cholesterol metabolism is self-evident.

PROCEDURE

The individuals used in this work were on a fairly standardized hospital diet. All were males. The age range was eighteen to thirty-five years. The body habitus was clinically determined as pyknic 5 and asthenic 5. The bloods were obtained in the morning, once a week, and while the individuals were in a fasting state. The method of estimation of the blood cholesterol was a modification of the Myers-Wardell[15] in which the total cholesterol was determined on whole blood. The procedure was carried out upon the blood immediately upon its withdrawal. Each cholesterol estimation was performed in duplicate, the average result being used in each instance and expressed as milligrams of cholesterol per 100 c.c. of whole blood. The normal range for this method as determined by Schube[16] and taking into consideration the error of the method, is 100 to 200 mg. of cholesterol per 100 c.c. of whole blood.

RESULTS

The cholesterol estimations were performed upon 160 samples of whole blood, one sample per week on ten individuals, over a period of four months. The changes in the concentration of the cholesterol in each person is shown in Table I. The total range, mean value, and the maximum and minimum deviation from that value are likewise shown in Table I.

The total cholesterol in the whole blood for the group of cases varied from 100 to 198 mg. with a mean value of 147 mg. This range and mean are entirely within the limits of normal for the method used, as Schube[16] has shown. Over the time period of four months used in this study, the blood cholesterol in the individual case was found to fluctuate in quantity somewhat. This fluctuation in successive weeks may be marked (73 mg. in Case 1, sixth to seventh week) or practically immeasurable (1 mg. in Case 10, eleventh to twelfth week). When the fluctuation of cholesterol is studied in the individual case as related to the mean value in that case, the fluctuation is less marked (from a maximum of +44 to –3 mg. to a minimum of +19 to ±0). If these values can be taken as representative, then it can be said that in the individual case the total blood cholesterol can vary in quantity from week to week when the differences of the successive values are studied, over a range of 0 to 73 mg., or over a lesser range of 19 to 47 mg. if each value is studied in relation to its individual mean value. Such variations still permit the cholesterol to remain within the limits of normal, for in no instance, regardless of fluctuation, did the blood cholesterol deviate from the range which was normal for the method.

DISCUSSION

This study exhibits several interesting facts: (a) When a large group of total cholesterols is estimated over a number of weeks in the bloods of normal individuals, the range of these values and the mean is within the normal range

TABLE I

TOTAL CHOLESTEROL IN MG. PER 100 C.C. WHOLE BLOOD

TIME IN WEEKS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	MEAN	DEVIATION FROM MEAN		RANGE	GENERAL MEAN
																		MAX.	MIN.		
Case 1	172	173	183	187	136	105	178	140	130	150	137	123	125	130	125	100	143	+44	-3	100-187	
Case 2	158	186	166	186	151	165	166	162	178	156	172	160	175	165	170	165	167	+19	-1	151-186	
Case 3	131	110	111	125	148	136	122	110	106	125	123	110	126	120	125	130	122	+26	±0	106-148	
Case 4	188	198	180	146	182	150	146	155	167	160	143	162	157	142	152	142	160	+38	±0	142-198	147
Case 5	148	138	136	120	151	130	120	136	125	126	132	133	137	120	140	132	132	+19	±0	120-151	(Range
Case 6	185	154	172	115	171	155	156	140	150	130	157	142	160	150	155	155	152	-37	+2	115-185	of
Case 7	155	145	157	150	170	140	135	145	148	143	154	153	145	140	145	150	148	+22	±0	135-170	Mean
Case 8	144	186	153	160	190	150	134	147	158	143	150	160	140	148	170	144	154	+36	-1	134-190	Values
Case 9	159	150	131	170	120	146	147	137	130	144	140	150	136	124	133	140	141	+29	-1	124-170	+13 to ±0)
Case 10	170	165	152	116	136	167	196	170	164	157	153	152	143	148	140	154	155	+41	-1	116-196	

and the mean range for total blood cholesterol as estimated by the given method. (b) When the total blood cholesterol is estimated and considered only in the individual case over a series of weekly intervals, the range continues within the limits of normal but the mean value for the individual may vary quite a bit. (c) When the range of total cholesterol in individual cases is compared, it is found that these individual ranges may vary markedly, although remaining within normal limits. (d) When the means of the blood cholesterol of the individuals are compared, it is found that there is a range of variation of 35 mg. (e) The age of the individuals in this series apparently did not affect the cholesterol, and (f) no correlation could be established between the body type and cholesterol nor variation of cholesterol. It is fully realized that for (e) and (f) the number of cases is small and that a greater number might show something of significance. It is the purpose here to present the factual observations.

Since it has been rather conclusively shown by Man and Gildea that in order to produce an alimentary lipemia, an excess of 200 gm. of fat must be taken by an individual at one meal, and since these individuals certainly never approached this figure in a total of three meals per day, it is highly improbable that the lipin in the food had anything to do with the variations observed. Furthermore, on a diet such as the above, Boyd, and Burger and Somach found that an increase in blood cholesterol which is significant does not occur. Therefore, such variations as have been found will have to be explained in some other way.

The difference in quantity of blood cholesterol in different normal individuals at the same time can only be explained at the present on the basis of individual peculiarities in handling the cholesterol in the blood, even though it be within normal limits at all times. The fluctuations in the blood cholesterol from week to week can be explained at the present only upon the same basis. It is highly probable that the ebb and tide of the blood cholesterol depend upon the needs of the body for it and upon the fluctuating ability of the organs of excretion to retain it in the blood stream. These, however, are merely conjectures and should, in our present state of knowledge of cholesterol metabolism, be regarded as such.

SUMMARY

In this study it has been shown that over a time period involving weeks:

a. The blood cholesterol in different individuals fluctuates along different patterns.

b. The blood cholesterol from week to week can change over a range of 0 to 73 mg.

c. The blood cholesterol when considered in relation to its individual mean over a long-time period fluctuates over a range of 19 to 47 mg.

d. There is certain ebbing and flowing of the blood cholesterol which is always within normal limits, which cannot be explained by any known mechanism, but which must be attributed to unexplained occasional changes in the fat metabolism of this or that person.

REFERENCES

1. Ahlfeld, F.: Zentralbl. f. Gynäk. **1**: 265, 1877.
2. Bloor, W. R.: J. Biol. Chem. **23**: 317, 1915.
3. Bloor, W. R.: J. Biol. Chem. **24**: 447, 1916; Physiol. Rev. **2**: 92, 1922.
4. Greenwald, L.: J. Biol. Chem. **21**: 29, 1915.
5. Reicher, K.: Verhandl. Kong. inn. Med. **28**: 327, 1911.
6. Burger, M., and Somach, I.: J. Biol. Chem. **97**: 23, 1932.
7. Chaikoff, I. L., McGavack, T. H., and Kaplan, A.: J. Clin. Investigation **13**: 1, 1934.
8. Gardner, J. A., and Gainsborough, H.: Biochem. J. **22**: 1048, 1928.
9. Hiller, A., Linder, G. C., Lundsgaard, C., and Van Slyke, D. D.: J. Exper. Med. **39**: 931, 1924.
10. Page, I. H., Pasternak, L., and Burt, M. L.: Biochem. Ztschr. **223**: 445, 1930.
11. Rony, H. R., and Levy, A. J.: J. Lab. & Clin. Med. **15**: 221, 1929.
12. Man, E. B., and Gildea, E. F.: J. Biol. Chem. **99**: 61, 1932.
13. Boyd, E. M.: J. Biol. Chem. **110**: 61, 1935.
14. Bloor, W. R.: Physiol. Rev. **2**: 92, 1922.
15. Schube, P. G.: J. Lab. & Clin. Med. **18**: 306, 1932.
16. Schube, P. G.: Am. J. Psychiat. **12**: 355, 1932.

STUDIES IN BACTERIOPHAGE*

III. The Significance of Tests for the Inhibition of the Bacteriophage Phenomenon by Human Serum

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INTRODUCTION

OUR combined clinical and laboratory experience with bacteriophage has shown us clearly that there is a definite relationship between the presence or absence of lysis in the test tube and the favorable or unfavorable action of bacteriophage in the patient.[1, 2] The treatment of certain cases yielding organisms resistant to bacteriolysis met with complete failure, while those which yielded susceptible cultures generally responded favorably. However, some of these cases failed to respond to the phage treatment. In an attempt to explain the discrepancies, we have studied the effect of the different substances, such as serum, pus, tissue extracts, etc., with which bacteriophage comes into contact when introduced into the lesion. The present paper concerns itself with the inhibition of bacteriophage by serum.

LITERATURE

Gratia[3] in 1921 observed that human serum contains a substance which neutralizes the lytic effect of bacteriophage on bacteria in the test tube and offered this fact as the possible explanation of certain failures in phage therapy. In 1925, however, Wolff[4] failed to confirm Gratia's findings. On the other hand, in 1928, Rosenthal[5] reported that he had found that the serum of patients suffering from recurrent furuncles or severe carbuncles, contained an inhibiting substance, demonstrable in dilutions as high as 1-2000, which was not found in the

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serum of persons free of infection. Later, Rosenthal[6] found that results obtained when he mixed serum with phage first and then added the bacteria were often different from those obtained when he mixed the serum with the bacteria first and subsequently added the phage. Inhibition of lysis sometimes took place by one method and not by the other. He concluded from these tests that in the first instance the serum directly neutralized the lytic agent while in the other it indirectly inhibited the lysis by rendering the bacteria resistant. The first effect he named "direct antiphage," the second, "indirect antiphage." Rosenthal stressed the importance of direct antiphage and suggested "autohemotherapy," stating that this caused the direct antiphage to disappear from the blood. However, he did not present any experimental data in substantiation of this statement.

In 1928, Raiga[7] reported 4 cases of failure of bacteriophage treatment in patients whose organisms were susceptible to the phage in the test tube, but whose serums were found to contain an inhibiting substance active in dilutions up to 1-2000.

In 1930, Raiga[8] enlarged upon his previous report and attempted to correlate the results of his therapy with the presence of the antiphage. This author followed Rosenthal in distinguishing two kinds of antiphages, calling them the antiphage of bacterial resistance (indirect of Rosenthal) and the antiphage of bacteriophage inactivation (direct of Rosenthal). After correlating his tests with the clinical results, Raiga, in contradistinction of Rosenthal, concluded that the antiphage of bacterial resistance (indirect) was of importance while the antiphage of bacteriophage inactivation (direct), which he encountered in practically every case, was of no significance. Beside the staphylococcus antiphage of bacterial resistance, Raiga found that many serums also had hemolytic streptococcus antiphage, which seemed to him to have considerable clinical importance. Raiga did not state his method for determining the presence of these antiphages in this report, but later publications indicate that he used the method of Rosenthal at the beginning of the study and that of Sertic later.[9-12]

In 1931, Riglieri and Fischer[13] studied 80 serums of surgical patients and found staphylococcus antiphage in every case.

In the same year, Applebaum and MacNeal[14] reported that purulent exudates, blood, and blood serum exerted a marked inhibitory influence on the lytic action of the staphylococcus bacteriophage. These authors were the first to suggest that any given serum might alter the lytic effect of phage in varying degrees when placed in contact with different strains of bacteria.

In 1931, Gratia and Mutsaars[15] and Mutsaars[16] extended their work and found that while serum had an inhibitory action on the phage, this action was usually transient, for lysis frequently took place in the second twenty-four hours. They also observed that a serum which prevented the action of the phage on *Staphylococcus aureus*, did not affect the action of phage on *Staphylococcus albus*.

Colvin[17] (1932) found that serum would sometimes inhibit the action of phage in high dilutions when the concentrated serum failed to inhibit.

How may the variable results of these different authors be reconciled? Differences in technic undoubtedly explain most of these variations, for there was no uniformity in the potency of the phages, the age or concentration of serum,

the temperature or duration of incubation, or the varying susceptibility of the different bacterial strains. Obviously complications are greatly multiplied when there are three variable factors. We have endeavored to minimize technical error and determine if possible the relative importance of the variable factors and estimate the clinical importance of this inhibiting substance.

TECHNIC OF THE TESTS

After due consideration we adopted the Rosenthal technic with the addition of separate phage and serum controls.

RESULTS

A. *With Respect to the Test Itself.*—We found that the results of the antiphage tests varied with the age of the serum, and that it was important to use serum not later than twenty-four hours after withdrawal. All tests had to be read at 18 to 24, 48 and 72 hours to avoid the possibility of falsely positive results. They were run at 37° C.

B. *With Regard to the Importance of the Different Variable Factors.*—

1. *The effect of different serums on a single phage in the presence of a single strain of bacteria:* In our earlier experiments we assumed from the reports of previous workers that the variable factors were in the serums rather than in the phage or in the bacteria. We therefore used a phage which we knew to be potent and a stock strain which was susceptible to the phage. Of 25 serums, 32 per cent yielded a positive test for direct antiphage, and 20 per cent a positive test for indirect antiphage.

2. *The effect of a single serum on different bacteriophages in the presence of a single strain of bacteria:* a. *Stock phages:* We tested a group of six serums with five different stock phages against a single strain of *Staphylococcus aureus.* With one of these phages, all but one of the serums gave strongly positive tests for both direct and indirect antiphage, while against the other five phages, five of the six serums gave either no reaction or a very weak antiphage action. It is evident, therefore, that the character and potency of the phage is an important factor in the antiphage tests.

b. *A comparison between stock phages and "specific" phages:* A group of 15 serums were tested for the presence of direct and indirect antiphage, using both a stock phage and an adapted phage (propagated at the expense of the patient's organism) with a single strain of susceptible bacteria. Of 15 serums tested with the stock phage, 80 per cent gave a positive test for direct antiphage, while only 60 per cent gave a positive test with the adapted phage. At first it was thought that the propagation of the phage in the presence of the patient's pus adapted the phage to the serum, but later it was found that some serums gave a positive antiphage test with the propagated phage, but not with the stock phage.

3. *The effect of different bacterial strains:* a. *Stock strains.* We studied the inhibitory action of six serums in the presence of six different strains of bacteria with the same strain of phage. From these tests, it was possible to conclude that the inhibition of lysis varied definitely with the microorganism used. Moreover, in this series there were normal serums of persons without concurrent staphylococcus infection which gave a positive antiphage test.

b. *A comparison of the homologous strains of bacteria with stock strains:* To find out whether another strain of bacteria could be used for the test instead of the homologous strain of the patient, we tested 21 fresh serums. With the stock strain, these serums gave 95 per cent positive tests for direct antiphage and 62 per cent for indirect antiphage. With the homologous strains, there were 17 per cent positive tests for direct antiphage and 47 per cent positive for indirect antiphage. These results show that a stock strain of bacteria cannot be used for the antiphage tests.

C. *The Clinical Significance of the Test.*—1. *A comparison of serums from normal individuals and serums from patients suffering from staphylococcus infections:* Thirteen normal serums and 34 patients' serums were tested. These two groups of serums yielded exactly the same percentage of positive direct antiphage tests, 80 per cent, but the group of serums from patients suffering from staphylococcus infections gave 35 per cent of positive indirect antiphage tests, while the normal serums gave 10 per cent. The same difference in indirect antiphage tests between normal and patients' serums was obtained when the homologous strain of staphylococcus was used instead of the stock strain. Thus we have a slight indication that indirect antiphage is associated with patients' serums more often than with serums of normal individuals.

2. *The positive test for antiphage in relation to the persistence of bacteriophage in the lesion:* In 23 cases, antiphage tests were made with the blood serum of the patient and the homologous bacterium. In these cases, also, the persistence of bacteriophage in the lesion was determined. In all but two of these cases, direct antiphage was present in the serum. Yet in 15 of these cases bacteriophage persisted in the lesions for forty-eight hours after its instillation. Indirect antiphage was present in 8 cases and in 15 it was absent. In 6 of the 8 cases having indirect antiphage in the serum, the phage persisted in the lesion for forty-eight hours, while in 5 of the 15 in which it was absent, the phage was retained in the lesion for only 24 hours.

It is obvious from these results that the antiphage demonstrated in fresh blood serum of patients with staphylococcus infections, which inhibits the action of phage in the test tube, does not seem to destroy the bacteriophage when it is instilled into the lesion.

ANTIPHAGE FINDINGS BY THE METHOD OF SERTIC

Having obtained certain data by the technic of Rosenthal which failed to confirm Rosenthal's opinion of the clinical significance of antiphage, we felt that this work would be incomplete unless we also used the more precise method of Sertic and Bulgakov.[18] But our results with Sertic's method ran practically parallel with our results with the Rosenthal technic. Lack of space prevents the publication of the details of these experiments.

DISCUSSION

Our study has confirmed the findings of earlier investigators who observed that the serum of human beings may possess an inhibiting action interfering with the lysis of bacteria by bacteriophage in the test tube. Of all the methods suggested, the most reasonable and exact are those of Rosenthal and of Sertic,

both of which were followed in this study. The demonstration of this inhibiting action is affected by a number of variable conditions which probably account for the varying results obtained by different observers.

The nature of this inhibition is not clear. With regard to the antiphage of phage inactivation (direct); if the phage is a ferment, the serum may absorb it, neutralize it, or provide a medium antagonistic to its action. If the phage is a living organism, the serum may destroy it in whole or in part, inhibit its growth, interfere with its metabolism, or divert its action from the bacteria to itself. With regard to the antiphage of bacterial resistance (indirect), there may be some alteration of the bacteria rendering them more resistant to the lytic action of the phage, the effect may be simply a mechanical protective coating or the serum may activate the bacteria to greater growth, which may in turn affect the lytic action of the phage.

The fact that fresh serum inhibits at first and as it gets older loses this power, indicates that the antiphage may be a very labile substance, possibly volatile, or a constituent of living protoplasm kept potent by living white cells or chemically active hemoglobin.

Our finding that the inhibition is more active at body temperature than at room temperature suggests that it might carry on this function in the patient. This is the temperature which favors bacterial growth, so that if there is any delay in the lytic action of the phage, the bacteria may get into an active growth phase and overwhelm the phage simply by force of numbers.

The evidence of inhibition in twenty-four hours, which is not present at forty-eight or seventy-two hours, may offer a clue as to the nature of the reaction. Here again the growth suggests a favoring of the metabolism of the bacteria and a temporary diversion of the phage from its lytic action. If clearing later takes place, it may be that the diversion is overcome by propagation of the phage or the inhibiting action of the serum may be exhausted or the active constituent may pass off from the serum just as it does in forty-eight to seventy-two hours in unused serum. A primary clearing with secondary growth might logically take place either from a neutralization or dispersion of the lytic power of the phage or delayed growth of those bacteria which survive the first onslaught of the lytic action.

The fact that normal serum possesses the inhibiting power to a less degree than patients' serums would seem to indicate that it is something engendered by the disease. However, it is difficult to reconcile its presence in some normal individuals, and its absence in a number of patients who have been suffering from furunculosis for years. The fact that the potency of the phage and the bacterial strain are as important factors in the result of the test as the serum itself, indicates that the reaction depends in large measure on the nature of the lytic property and on the growth, metabolism and physical properties of the bacteria themselves. If the antiphages of the serum actually inhibited the action of phage in the lesion, as Rosenthal and Raiga have maintained, one would expect that the bacteriophage would be destroyed in a shorter length of time after insertion into the lesion than when antiphages were not present. This cannot be demonstrated, however. We have studied the problem from every conceivable angle and have found no evidence whatsoever of its clinical significance.

SUMMARY

1. The serum of the majority of human beings possesses an inhibiting action, interfering with the lysis of *Staphylococcus aureus* and *Streptococcus hemolytic* by their corresponding bacteriophages in the test tube.

2. We have demonstrated this phenomenon first by the method of Rosenthal and later by the more precise method of Sertic.

3. This inhibiting substance can be best demonstrated in fresh serum at body temperature after twenty-four hours of incubation.

4. The result of the test depends upon the strain of bacteria used for the test and the strain and the potency of the bacteriophage, as well as upon the serum itself.

5. In this inhibition of lysis, the serum may affect the bacteriophage directly (antiphage of phage inactivation), or it may have an indirect action by rendering the bacteria resistant (antiphage of bacteria resistance). The serum of some individuals may harbor both antiphages.

6. The antiphage of bacteriophage inactivation (direct) was present in 80 per cent of both normal persons and those suffering from furunculosis. Antiphage of bacterial resistance was present in 35 per cent of infected individuals and in 18 per cent of normal persons.

7. No correlation was found between the presence of antiphage in the serum of the patient and the result of bacteriophage treatment by the direct introduction of bacteriophage into the lesion.

8. The presence of antiphage in the serums did not affect in any demonstrable degree, the bacteriophage administered directly into the lesion of the patient.

9. The presence or absence of these inhibiting substances in the serum of patients is apparently of no clinical significance in the treatment of local staphylococcus infections.

REFERENCES

1. Jern, H. Z., Howes, E. L., and Meleney, F. L.: Studies in Bacteriophage. The Behavior of the Bacteriophage and the Bacteria in the Lesion After the Treatment of Acute Staphylococcus Skin Infections With Bacteriophage, J. Lab. & Clin. Med. **19**: 1257, 1934.
2. Jern, H. Z., and Meleney, F. L.: Methods of Maintaining and Testing the Potency of Bacteriophage, J. Lab. & Clin. Med. **20**: 862, 1935.
3. Gratia, A., and Jaumain, D.: Identité du phénomène de Twort et du phénomène de d'Herelle, Compt. rend. Soc. de biol. **85**: 880, 1921.
4. Wolff, L.: Bacteriophage Action in Blood, Nederl. tijdschr. v. Geneesk. Amst. **2**: 1088, 1925; Abst. J. A. M. A. **85**: 1440, 1925.
5. Rosenthal, P.: Contribution à l'étude des antiphages, Compt. rend. Soc. de biol. **99**: 1211, 1928.
6. Rosenthal, P.: Contribution à l'étude des antiphages, Compt. rend. Soc. de biol. **100**: 1019, 1929.
7. Raiga, A.: Une cause d'insuccés du traitement par le bactériophage de d'Herelle, J. de Practiciens **48**: 794, 1928.
8. Raiga, A.: Les anti-phages dans la bactériophagy, Compt. rend. Soc. de biol. **104**: 745, 1930.
9. Raiga, A.: Traitement des infections staphylococciques par le bactériophage de d'Herelle, Revue Med. Univ. 4 and 5, 1930.
10. Raiga, A.: Guérison par une inoculation intraveineuse de bactériophage, d'une pyohemie a staphylocoque, Bull. et mém. Soc. de Chir. de Paris, Seance du 1-er Juin, 1934.
11. Raiga, A.: Le rôle de terrain organique dans l'evolution et le traitement de la furonculose. Antiphages et Hyperglycémie, Bull. et mém. Soc. de Chir. de Paris 26, No. 13, Seance 19, October, 1934.

12. Raiga, A.: Guérison d'une arthrite purulente de genou à Streptocoque et tétragène par une inoculation intra-articulaire de bactériophage, Bull. et mém. Soc. de chir. de Paris **26**: No. 12, 1934.
13. Riglieri, P., and Fischer, I.: Contribution à l'étude des propriétés anti-bactériophagiques des serums humains, Compt. rend. Soc. de biol. **108**: 674, 1931.
14. Applebaum, M., and MacNeal, W.: The Influence of Pus and Blood on the Action of Bacteriophage, J. Infect. Dis. **49**: 225, 1931.
15. Gratia, A., and Mutsaars, W.: L'action inhibitrice du sérum normal sur la lyse du staphylocoque doré par les bactériophages staphylococciques polyvalents, Compt. rend. Soc. de biol. **106**: 943, 1931.
16. Mutsaars, W.: De l'action protectrice exercée par le sérum normal sur les staphylocoques dorés contre la fixation du bactériophage, Compt. rend. Soc. de biol. **108**: 235, 1931.
17. Colvin, M. G.: Behavior of Bacteriophage in Body Fluids and in Exudates, J. Infect. Dis. **51**: 527, 1932.
18. Bulgakov, N. A.: Personal Communication.

THE DOCTOR AS SCHOLAR

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THE contributions of the medical man to pure scholarship have been of the highest order. Together with the doctor of divinity the doctor of medicine helped to keep the flame of learning alive during the Middle Ages, and in the centuries following added much to its luster by many significant contributions to almost every branch of pure scholarship.

Among the first of the medical scholars was Thomas Linacre who lived from 1460 to 1524. He began his education at Oxford University and continued it at the ancient University at Padua, Italy, from which he received his degree in medicine. Upon his return to England he distinguished himself as a physician, and when Henry VIII became king, Linacre was appointed as one of his physicians. Dr. Linacre enjoyed a large medical practice in London and numbered many prominent persons as his patients.

But medicine was not the only thing that interested Linacre. He was very much interested in religion and received priest's orders. He then restricted his medical work and devoted more of his time to scholarship. He was the founder of the College of Physicians which was incorporated in 1518. Linacre was its first president, and he retained this office until his death. He aided the Universities of Oxford and Cambridge in scholarly research as well as in the matter of monetary gifts. He was one of the first of the great English scholars.

A contemporary of Linacre was John Caius (1510-1573) who was born at Norwich and studied at Gonville Hall, Cambridge. Like Linacre he went to Padua to round out his education. Here he came in contact with the leading medical lights of the time, Montanus in medicine and Vesalius in anatomy. He became a very close friend of the great anatomist and lived in his house for eight months.

His career seemed to parallel that of Linacre with remarkable fidelity. Upon his return to England he became physician to Edward VI and later to Queen Mary. His practice was enormous and he became wealthy. He never married, and for this reason determined to devote his wealth for the encouragement of scholarship. In 1557 he obtained royal permission to refound Gonville

Hall, his Alma Mater, and the institution now became known as Caius College, which name it retains to the present day. Two years before his death he was elected President of the College of Physicians for the ninth time. He carried forward the glorious traditions of his illustrious predecessor and founder of the College, Thomas Linacre.

In the year 1559 Dr. Caius was persuaded to accept the Mastership of the College, made vacant by the death of Thomas Bacon. Henry VIII had freed England from the Catholic Church, but there were many who still adhered to the Pope; Dr. Caius was suspected of being one of these, and for this reason he was not very popular with the Fellows of the college.

The massacre of St. Bartholomew aroused indignation all over England against those with Catholic leanings, and a raid was made on Caius' house where a collection of ornaments used in Catholic rituals was discovered. His life thereafter was far from happy. He contributed much to the learning of that period, particularly to natural history.

Among the earlier of the classical scholars was Richard Mead (1673 to 1754), who studied classical literature and antiquities at the University of Utrecht, and later medicine at the University of Leyden, where a countryman, Archibald Pitcairne, was Professor of Physic. Upon receiving his medical degree he returned to England where he practiced at Stepney and became physician to the world-famous St. Thomas' Hospital. Dr. Mead was an accomplished classical scholar, and in spite of his enormous practice, found time to encourage classical studies and helped in more practical ways by constantly presenting large sums of money to promote classical scholarship.

A medical man who attained great prominence as a Greek scholar was Francis Adams (1796 to 1861). He was born at Lumphanan, Aberdeenshire, graduated in arts and studied medicine at Aberdeen. Then he went to London where he received his M.R.C.S. Upon graduation he settled down in Banchory where he spent the rest of his life.

Dr. Adams was very much interested in pure classical scholarship and translated many of the Greek writings into English. He was among the first of the doctor-scholars to render the Greek physicians, Hippocrates, Paulus Aegineta and Aretaeus, into English. His fame as a classical scholar began to attract attention, and he was offered the Chair of Greek in his own university. His love for medicine was so great that he could not bear the idea of giving up medical practice, and for this reason refused the proffer.

John Freind (1675-1728) was another physician who attracted wide attention by his fine classical scholarship. He received his academic and medical education at Oxford University. In 1704 he lectured on chemistry at his university and in the following year went to Spain as physician to the British forces. In 1712 he became an F.R.S. and ten years later became a member of Parliament for Launceston.

Soon after he became implicated in Bishop Atterbury's plot for the restoration of the Stuart family and was committed to the Tower of London in 1723 on a charge of high treason. Here he remained for three months, devoting his time to studies in Latin. It is said that he owed his freedom from the Tower to Dr. Mead, who, when summoned to attend Sir Robert Walpole, refused to prescribe for him until he promised to set Freind at liberty.

One of the earliest contributions to literary scholarship was made by Robert Watt (1774-1819). He was born on a farm near Stewarton in Ayrshire and graduated in arts at Glasgow. For awhile he acted as parochial schoolmaster, but he was very much interested in medicine and returned to Glasgow to enter upon his medical studies. In 1799 he became a Licentiate of the Faculty of Physicians and Surgeons. In the same year he began general practice in Paisley where he remained for eleven years. In 1810 he obtained his M.D.

Dr. Watt was a promoter and the first president of the Glasgow Medical Society. In 1816 he was elected president of the Glasgow Philosophical Society, and in the following year his health became so impaired that he withdrew from medical practice.

He then left Glasgow for Campvale where he entered upon his studies in literature. Here he conceived and carried out his great work the *Bibliotheca Britannica*. His health grew steadily worse and he died in 1819, with the Bibliotheca practically completed.

Dr. Watt's great achievement, the *Bibliotheca Britannica; or a General Index to British and Foreign Literature*, was completed five years after his death. It was a monumental achievement for one man. It is the first great bibliographic work of modern times. It consists of four large quarto volumes. The first two contain the names of more than 40,000 authors, with brief summaries of their works. The second part contains all the books mentioned in the first part, arranged under subjects.

Another doctor who contributed much to literary scholarship was Peter Mark Roget (1779-1869), the son of a French pastor in London. He graduated in medicine at the University of Edinburgh at the early age of nineteen, but he continued his studies in London.

In the year 1805 Dr. Roget was appointed a physician to the Manchester Infirmary, and in this capacity he also became one of the founders of the Manchester Medical School. Three years later he removed to London where he engaged not only in a very extensive medical practice but in other activities as well.

In between office consultations and calls at his patients' homes, Dr. Roget made a very thorough investigation of the water supply of the metropolis at the request of the government. He also invented a logo-logarithmic slide rule which won for him election to the Royal Society. In time he succeeded Sir John Herschel as secretary of this society. During this time he also wrote a Bridgewater treatise on *Animal and Vegetable Physiology Considered With Reference to Natural Theology*. This work attained great popularity and went through many editions. Dr. Roget was also one of the founders of the University of London.

The general public remembers Dr. Roget not for the many accomplishments during his lifetime, but for one single literary production. This is the world-famous *Roget's Thesaurus of English Words and Phrases*. He wrote this book after 1840 when he retired from professional practice to devote himself to this work exclusively.

Roget's *Thesaurus* went through 28 editions during his lifetime, and what edition it has reached at this date, almost a hundred years after it was first published, I do not at the moment know. It has been a steady seller and is still as popular as ever. There is not a writer or editor of English anywhere in the world who has not heard of this work or who has not a copy on his desk.

William Alexander Greenhill (1814-1894) was another medical man who attained great eminence as a scholar. He was born in London and received his medical education at the Universities of Oxford and Paris. He set up in medical practice at Oxford, and devoted all his spare time to the study of Greek and Arabic. Among his numerous contributions to classical scholarship were a Greek and Latin edition of the *Physiology of Theophilus,* a Latin edition of Sydenham's works and an English translation of the works of Rhazes from the Arabic. He also contributed many articles in Smith's *Dictionaries of Greek and Roman Antiquities and Biography.*

Sir Hans Sloane (1660-1753) is as well known as a scholar as a physician. He was born in County Down, and studied at Paris and Montpellier. In 1867 he went as physician to the West Indies, and in time became the personal physician of the Governor of Jamaica. While in the West Indies, he undertook a systematic study of the flora and fauna of that part of the world, collecting many specimens which he brought back to England.

Upon his return to his native country he settled in Bloomsbury Square and in time acquired a large practice. He numbered many prominent people among his patients, Queen Anne among them. He became one of the most illustrious members of the Royal Society and in 1727 he succeeded Sir Isaac Newton as president which post he held until 1741.

Dr. Sloane was very generous in his bequests to the needy. Throughout his life Sir Hans was a tireless collector of valuable specimens of natural history and after his death his famous collections were used in starting the British Museum, which was opened to the public in 1759.

One of the most famous of modern Orientalists was a medical man, Edward Granville Browne (1862-1926). He studied medicine at the University of Cambridge from which he received his M.B. Later he was elected a F.R.C.P., but he never practiced medicine. He was more interested in oriental studies than he was in medicine. He had a thorough knowledge particularly of Persian and Arabic. He returned to his university in a teaching capacity, but not in medicine. He became lecturer in Persian, and he held this position with distinction for many years. In 1902 he was appointed Sir Thomas Adams Professor of Arabic at the University of Cambridge.

To chemical scholarship doctors have made many important contributions. It was a physician, Dr. Thomas Andrews, who was the first to demonstrate the true nature of ozone, proving it to be an allatropic form of oxygen. Joseph Black is immortalized as the discoverer of latent heat in 1761. Dr. Black also originated the theory of specific heat. In 1767 he made the first attempt to inflate a balloon with hydrogen.

Another notable chemist was Daniel Rutherford (1749-1819) who was born in Edinburgh and who received his medical education at the university in that city. His thesis for the M.D. degree in 1772 was significant because of the clear distinction he made between carbonic acid and nitrogen. He was also the first to isolate the gas nitrogen by burning substances in an enclosed volume of air and absorbing the carbonic acid thus formed.

The contributions of medical men to scholarship in many different fields have been of the greatest importance for a great many years. There is scarcely a branch of scholarship which has not felt the hand of the physician.

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LABORATORY METHODS

A PRECISE SILVER IMPREGNATION METHOD FOR BLOOD CELLS*

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MANY problems in hematologic morphology and differentiation remain unsolved because of limitations in our technichal methods. Similar problems in other fields of histology have been cleared up by the use of silver impregnation methods. It is, therefore, surprising to find that silver impregnation has had only limited application in the field of hematology.

Rhinehart[1, 2] impregnated erythrocytes with silver and studied their hemoglobin content. His method also is suitable for the impregnation of nucleated blood cells, but the technic is too complicated to lend itself to wide application. Dawson[3] applied the formulas devised by Cajal and da Fano, to the study of the reticular granules and segregation apparatus of erythrocytes. These methods, however, are not sufficiently exact to yield standard results. Tomita, Kawabe and Yuba[4] and Perez Ara[5] also used silver to impregnate blood cells, but their reports are not available to me. The following method has been in use for some time. It is relatively simple and yields uniformly precise results.

METHOD

Preparation.—Blood films, bone marrow smears or "touch preparations" from marrow, spleen, or lymph nodes are made in the usual manner and dried in air. These may be stored for an indefinite period, if kept free of dust, without impairment of the subsequent impregnation. Because cover slips can be stored in small cardboard containers which are easily numbered, it is more convenient to make the smears on cover slips than on slides.

Fixation.—The preparations are fixed in 10 per cent formalin (4 per cent formaldehyde) which need not be neutralized. Fixation is complete in a few minutes and is followed by a wash in water.

Mordanting.—The fixed smears are mordanted in 2.5 per cent aqueous solution of ferrous ammonium sulphate (iron alum) for ten minutes. Mordanting may be prolonged almost indefinitely without apparent interference with the subsequent impregnation. The mordant is followed by thorough washing in four changes of distilled water.

Coating.—The cover slips are dipped in 1.0 per cent aqueous solution of gelatin, to 50 c.c. of which are added two drops of 2 per cent aqueous sodium carbonate. This protects the smears during impregnation and prevents the deposition of diffuse silver precipitate while the preparations are in the silver

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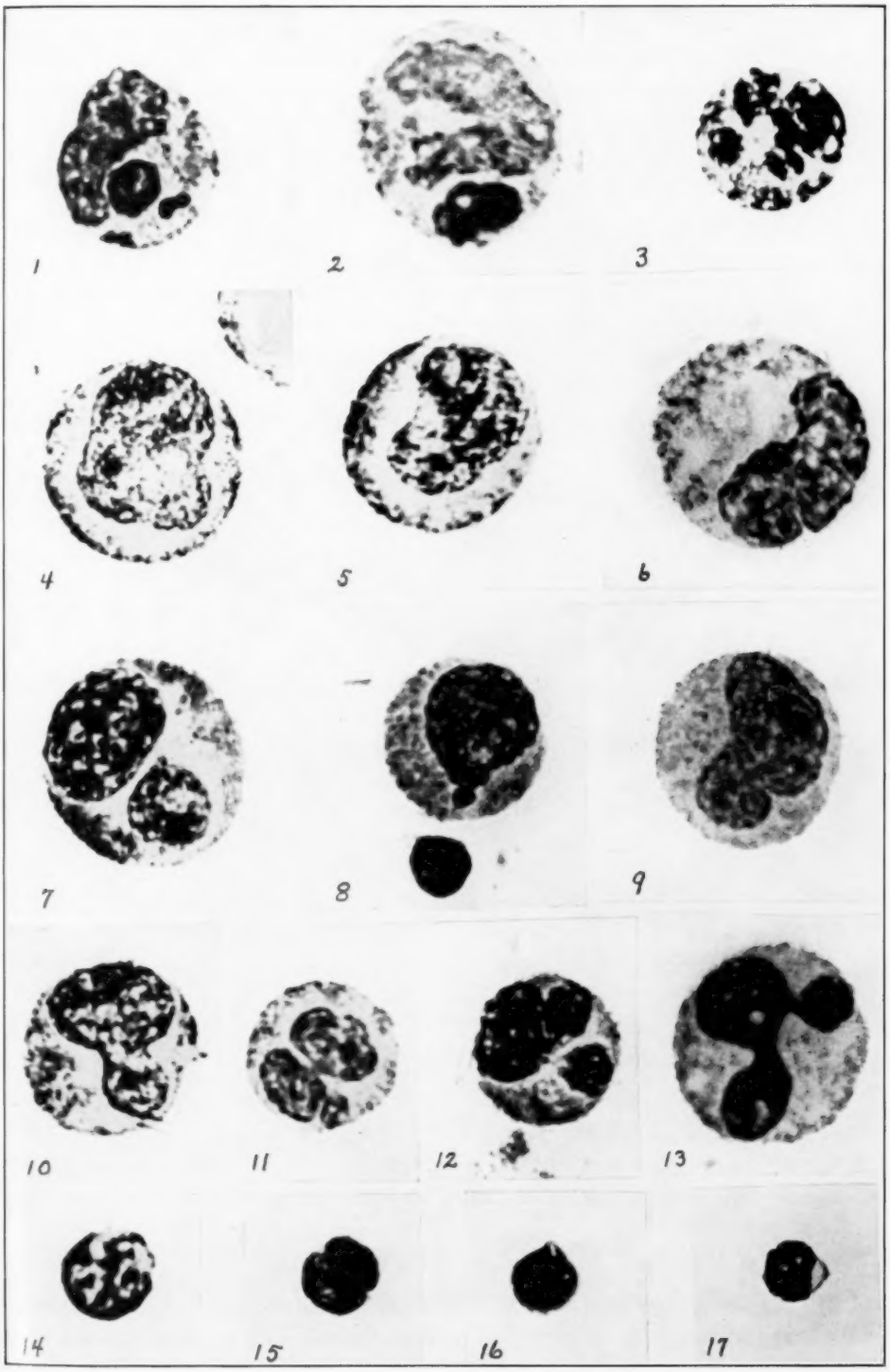

Fig. 1.—*1* and *2*, Hemohistiocytes containing phagocytosed normoblasts ×1700. *3*, Hemohistiocyte containing fragmented normoblasts ×1300. *4* and *5*, Myeloblasts ×1700. *6*, Premyelocyte ×1700. *7*, Young myelocyte ×1700. *8*, Myelocyte and intermediate normoblast ×1300. *9* and *10*, Metamyelocytes ×1700. *11* and *12*, Young polymorphonuclear leucocytes ×1300. *13*, Polymorphonuclear leucocyte ×1700. *14*, Macronormoblast ×1300. *15*, Intermediate normoblast (dividing) ×1300. *16* and *17*, Micronormoblasts ×1300.

bath. The excess of gelatin is allowed to drain off and the cover slips are briefly dipped in distilled water. This washes away some of the gelatin, leaving only a thin film.

Impregnation.—The diammoniosilver hydroxide solution of Kubie and Davidson[6] diluted with an equal amount of distilled water is employed:

To 5 c.c. of 10.2 per cent aqueous solution of silver nitrate, add strong ammonium hydroxide solution until the precipitate of silver hydroxide is just dissolved. Then add 5 c.c. of 3.1 per cent aqueous solution of sodium hydroxide and just dissolve the resultant precipitate with a drop or two of strong ammonium hydroxide. Dilute the diammoniosilver hydroxide solution to 100 c.c. with distilled water. If kept in a well-stoppered brown bottle, this solution will keep for a week or two. The smears are impregnated for five to fifteen minutes, using

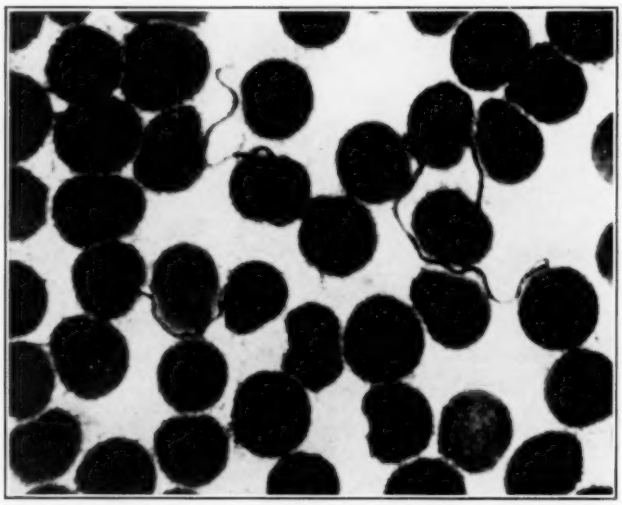

Fig. 2.—Spirillum Recurrentis. Blood film of mouse experimentally infected with African recurrent fever. Magnification ×2000 (approx.).

a covered dish. Following impregnation, the preparations are rapidly washed in hot distilled water (60° C.) to remove the gelatin.

Reduction.—The silver is reduced in alum-formol solution: 10 per cent formalin 90 c.c. and 2.5 per cent iron alum 10 c.c. Reduction is complete within a few seconds and is followed by a thorough wash in water to remove all trace of alum. The smears are then dehydrated in alcohol, cleared in xylol and mounted in balsam.

RESULTS

All the cells are impregnated. The erythrocytes have a finely granular cytoplasm and take a faint brown color. Some of the erythrocytes stain more intensely and have a coarsely granular, slightly reticulated cytoplasm. These probably are young red blood cells. The nuclei of the normoblasts are heavily impregnated and appear black. The cytoplasmic ring surrounding the nucleus of the micronormoblast takes the light brown color of the mature erythrocyte.

The nuclear pattern of the normoblast is very clearly apparent. The granular white blood cells are just as easily recognizable as the erythrocyte series, so that all stages of maturation from myeloblast to adult polymorphonuclear is easily identified. Nuclear and cytoplasmic granules also are precisely impregnated and, in all, a sharply defined picture of cell size is presented by this method. After a little experience, the other definitive cells of bone marrow or peripheral blood are also easily recognized.

COMMENT

An important feature of the method is its lability. For this reason the time periods for the various steps are not absolute. As with almost all staining methods, so with this—the technic, once mastered, may be modified to suit individual taste. The period of impregnation may be lengthened considerably, without danger of overimpregnation, if the wash following impregnation is lengthened proportionately. The gelatin film is not readily soluble and a thin film persists. Addition of sodium carbonate to the gelatin renders the latter almost transparent, so that the cells stand out in bold relief. The alum-formol reducing solution has proved more selective than simple formalin. The alum mordant may be used repeatedly. The solutions are easy to prepare and the resultant picture usefully supplements the aniline dyes in common use.

CONCLUSION

A simple method is described for the precise silver impregnation of blood films, bone marrow smears, or "touch preparations" from spleen or lymph nodes.

The method may be outlined as follows:

1. Smear preparations of bone marrow, blood films, or "touch preparations" are dried in air and fixed in 10 per cent formalin.
2. Wash in water.
3. Mordant in 2.5 per cent aqueous solution of iron alum for 10 minutes or longer.
4. Wash in four changes of distilled water.
5. Dip in 1.0 per cent aqueous solution of gelatin to which is added a drop or two of 2 per cent sodium carbonate, and drain.
6. Wash quickly in distilled water.
7. Impregnate in diammoniosilver hydroxide solution for five to fifteen minutes.

To 5 c.c. of 10.2 per cent silver nitrate add strong ammonia, drop by drop, until the precipitate is dissolved. Add 5 c.c. of 3.1 per cent sodium hydroxide and redissolve the precipitate with strong ammonia. Dilute to 100 c.c. with distilled water.

8. Wash in distilled water, heated to 60° C.
9. Reduce in alum-formol. Ten per cent formalin 90 c.c. and 2.5 per cent iron alum 10 c.c.
10. Wash in tap water.
11. Dehydrate in alcohol, clear in xylol and mount in balsam.

REFERENCES

1. Rhinehart, J. F.: Unusual Structures in the Erythrocyte. II. A Precise Nuclear Impregnation Method, Anat. Rec. **52**: 151, 1932.
2. Rhinehart, J. F.: Intracellular Precipitation and Impregnation of Hemoglobin in Erythrocytes, Folia haemat. **48**: 231, 1932.
3. Dawson, A. B.: Further Study of Reaction of Amphibian Erythrocytes to Vital Dyes, Osmic Acid and Silver Salts With Special Reference to Basophilia and Reticulation, Anat. Rec. **42**: 281, 1929.

4. Tomita, T., Kawabe, H., and Yuba, S.: Phylogenetic and Ontogenetic Studies on Finer Structures of Erythrocytes in Vertebrates by Means of Silver Impregnation Methods, Folia anat. japon. **12**: 129, 1934.
5. Perez Ara, A.: New Technic for Staining of Blood Films by del Rio Hortega Method, Rev. de Med. y cir. de la Habana **38**: 691, 1933.
6. Kubie, L. S., and Davidson, D.: The Ammoniacal Silver Solutions Used in Neuropathology, Their Staining Properties, Chemistry and Methods of Preparation, Arch. Neurol. & Psychiat. **19**: 888, 1928.

A SIMPLE ETHER ANESTHESIA APPARATUS FOR EXPERIMENTAL ANIMALS*

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IN BACTERIOLOGIC experimentation on the mammalian fetus we[1] have had repeated occasion to submit pregnant animals to surgical procedures. The problem of suitable anesthesia has been an important one in this work. It was necessary to have a drug which would not predispose to abortion following operative manipulation of the gravid uterus, and which would not impair the vitality of the fetuses. At the same time it was desirable that the method of administration be simple and if possible under the control of the surgeon. Various basal anesthetics such as urethane and barbiturates, either alone or accompanied by local anesthesia, were found to depress the metabolism of the animals greatly when given in amounts sufficient to produce surgical relaxation, and often confused the results of experimental fetal inoculation. Narcosis effected by dropping ether from a separatory funnel onto a mask gave better results, but still left much to be desired. More recently a simple apparatus for the administration of ether vapor has been devised and found very effective. Although such an apparatus may be in use elsewhere, we have not seen it described. Accordingly we take this means of calling it to the attention of others who may be interested.

A moderate and constant stream of compressed air, either from a central supply or from a tank, is admitted into rubber tubing (Fig. 1). This stream is divided by a Y tube and connections to pass through water and ether bottles in parallel. These may be ordinary glass bottles with two-hole rubber stoppers, as illustrated, but the usual gas washing bottles with glass stoppers are better because ether vapor slowly digests a rubber stopper. The bottles are taped together to make them more stable. The bottle containing water should be about half full, the ether bottle about one-fourth full. The divided currents of air, carrying water vapor and ether vapor respectively, are reunited by a Y tube and connections, and the final mixture is released through a glass cylinder into which the head of the animal is inserted. The glass cylinder has an outlet orifice of a size sufficient to permit loose insertion of the animal's head and a free flow of the gaseous mixture. Different outlets are used for animals of different sizes, such as mice and guinea pigs.

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The proportion of ether vapor and air in the final mixture is controlled by the one screw clamp on the ether line. If this is opened widely, all of the air current passes through the ether bottle, due to the lesser hydrostatic pressure of the ether, thus providing a maximal ether vapor content. Partial closure of the clamp divides the stream between the two bottles, and the relative flow through each may be judged by the relative rate of bubbling. The mixture may be changed almost instantly to one of pure air by closing the clamp. Flowmeters may be inserted in the air and ether lines for quantitative control of the mixture, but we have not found this refinement essential.

The apparatus offers the advantages of simplicity, accurate and delicate control of the ether vapor-air mixture under direct visualization and by means of

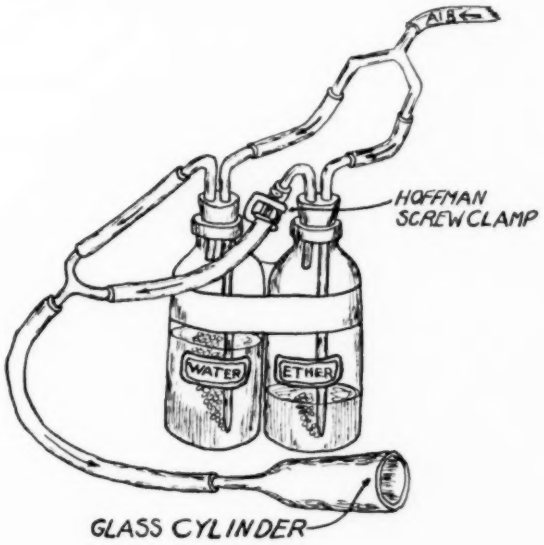

Fig. 1.

a single screw clamp, a constant supply of fresh air with the ether vapor, and continuous visual observation of the animal's condition through the glass cylinder. The screw clamp may be sterilized or covered with sterile gauze, so that regulation of the anesthesia may be in the hands of the surgeon. We have employed the apparatus only for mice, guinea pigs, and rabbits, but it should be adaptable to any experimental animal. Its use has greatly facilitated our experimental work. Anesthesia accidents have been practically eliminated and abortions following operative procedures have been considerably reduced.

REFERENCE

1. Woolpert, O. C.: Direct Bacteriological Experimentation on the Living Mammalian Fetus, Am. J. Path. 12: 141, 1936.

MODIFICATIONS IN THE EAGLE ANTIGENS FOR USE IN THE COMPLEMENT FIXATION AND FLOCCULATION TESTS FOR SYPHILIS, AND MINOR CHANGES IN THE TECHNIC OF THESE TESTS*

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A HIGHLY sensitized antigen originally described for use in a flocculation test was subsequently found to yield sensitive and specific results in a four-hour ice box Wassermann technic described in this JOURNAL in 1934.[2] As originally described, the antigen was a 95 per cent alcoholic extract of beef heart, fortified with 0.6 per cent each of cholesterol and corn germ sterol. It has since been found that the antigen is significantly improved by using absolute instead of 95 per cent ethyl alcohol. In the flocculation test, a troublesome crystallization of some of the corn germ sterol, when the antigen is stored at room temperature, is eliminated. More important, the difference between the opalescence of the negative result, and the white aggregates floating in clear serum which constitute a positive result, becomes more sharply defined.

In the Wassermann test, the use of absolute alcohol makes it possible to sensitize the extract with 1 per cent of cholesterol, and thus to eliminate the use of corn germ sterol for that reaction. The antigen dilution formed by adding 120 volumes of salt solution to one volume of antigen is homogeneous and opalescent, unlike the finely granular suspension of crystals previously formed by the antigen containing both cholesterol and corn germ sterol; and negative reactions are no longer obscured by the fact that the fine crystals of sterol may simulate a haze of undissolved cells. Moreover, unlike the doubly sensitized strably anticomplementary under the conditions of the test to be described. Finally, the antigen containing 1 per cent cholesterol is just as sensitive as the antigen containing 0.6 per cent each of corn germ sterol and cholesterol.

Several other minor modifications have been made in the Wassermann and flocculation technics as originally described. These changes are italicized in the following brief description of the tests.

A. PREPARATION OF THE BASIC EXTRACT FOR USE IN BOTH THE WASSERMANN AND FLOCCULATION TESTS

Fifty grams of dried powdered beef heart (Difco) are extracted for fifteen minutes at *30° to 37° C.* with *250 c.c.* anesthesia ether, with frequent shaking. The mixture is filtered with suction, the ether extract is discarded, and the powder is similarly extracted with a second portion of fresh ether (250 c.c.).

*From the Department of Bacteriology, School of Medicine, University of Pennsylvania. Received for publication, March 2, 1936.

This is repeated for a total of *four* extractions. All the ether extracts are discarded. The beef heart powder is then washed on the filter with 100 c.c. of fresh ether, thoroughly dried, and extracted with 250 c.c. of *absolute* ethyl alcohol for three to five days at 20° to 37° C. At the end of this time, the alcohol mixture is filtered, and the moist powder is washed with small portions of fresh absolute alcohol until the combined alcoholic extract and washings measure 250 c.c.

For use in the flocculation reaction this basic extract is fortified as heretofore with 0.6 per cent each of cholesterol and corn germ sterol (6 mg. of each sterol per c.c. antigen). For the Wassermann test, the extract is fortified with *1 per cent cholesterol* (10 mg. per c.c. antigen) but no corn germ sterol. In both cases, the required amount of sterol is added to a measured volume of antigen, and is dissolved by boiling.

B. TECHNIC OF WASSERMANN TEST

1. *Antigen.*—a. The antigen is *anticomplementary* in a 1:3 or 1:6 dilution, and is not significantly more hemolytic than pure alcohol. Every lot of antigen should be tested once for these undesirable properties by the technic described in Tables I and II.

TABLE I

ANTICOMPLEMENTARY TITRATION OF ANTIGEN

	ANTIGEN DILUTION*						
	1	1:2	1:3	1:4	1:6	1:8	1:12
Antigen dilution, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.85 per cent salt solution, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Complement, 1:10, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
After 4 hours at 0° to 5° C., followed by ½ hour at 37° C., add 0.8 c.c. of sensitized cells to all the tubes.							
Example of reading of hemolysis after ½ hour at 37°	complete	0	0	partial	complete	complete	complete
Conclusion: Antigen is anticomplementary up to a 1:4 dilution.							

*These dilutions are readily prepared as follows:

Antigen, c.c.	0.4	0.2	0.13	0.1	0.07	0.05	0.033
Salt solution, c.c.	0	0.2	0.25	0.3	0.35	0.35	0.35
Final dilution of antigen	1	1:2	1:3	1:4	1:6	1:8	1:12

TABLE II

HEMOLYTIC TITRATION OF ANTIGEN

	ANTIGEN DILUTION†						
	1	1:2	1:3	1:4	1:6	1:8	1:12
Antigen dilution, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.85 per cent salt solution, c.c.	0.8	0.8	0.8	0.8	0.8	0.8	0.4
Cell suspension,* c.c.	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Hemolysis is read after ½ hour at 37° C.							
Example of reading of hemolysis	complete	none	none	none	none	none	none
Conclusion: The antigen is not significantly hemolytic.							

*Can be sensitized or unsensitized, but should be 1½ per cent by volume.
†See footnote to Table I for method of preparing these dilutions.

b. *The optimum antigen dilution* is that which detects the smallest quantity of reagin, and should be determined once for every lot of antigen by the technic described in detail elsewhere[3] and illustrated in Table III. The optimum dilution is usually between 1:100 and 1:200, and a 1:120 dilution can be used routinely as a reliable mean.

TABLE III

METHOD OF DETERMINING THE OPTIMUM ANTIGEN DILUTION

ANTIGEN DILUTION USED IN TEST	POSITIVE SERUM	RESULT OF WASSERMANN TEST* WITH SAME SERUM DILUTED† 1:2	1:4	1:8	1:16	1:32	1:64
1:40	+	+	+	±	0	0	0
1:80	+	+	+	+	±	0	0
1:100	+	+	+	+	+	0	0
1:120	+	+	+	+	+	0	0
1:160	+	+	+	+	+	0	0
1:200	+	+	+	+	±	0	0
1:400	+	+	+	±	0	0	0

Conclusion: The optimum dilution, that which detects the smallest amount of reagin, is approximately a 1:120 dilution.

*Same technic as that to be described.

†These dilutions can be readily prepared in sufficient quantity for the entire experiment by the following set-up:

Positive serum, c.c.	4	2	1	0.5	0.25	0.125	0.062
Salt solution (or negative serum)	0	2	3	3.5	3.75	3.9	4.0
Final dilution of positive serum	1	1:2	1:4	1:8	1:16	1:32	1:64

c. *Method of dilution:* The required amount of 0.85 per cent salt solution (e.g., 120 volumes) is poured *slowly* into one volume of antigen. The dilution should be opalescent, but homogeneous, and should contain no visible granules. Two-tenths cubic centimeters of antigen usually suffice for 50 routine serum tests.

2. *Preparation of Human Serum and Spinal Fluid.*—The clear serum is inactivated at 56° C. for twenty minutes. The *native amboceptor* for sheep red blood cells often present in human serum can be removed by adding 0.1 c.c. of a 50 per cent washed suspension of sheep cells to 1 c.c. of inactivated serum, and centrifuging out the cells after fifteen minutes at room temperature. An alternative method is to add the sheep cells to the whole human blood, and centrifuge after fifteen minutes in the ice box. The latter procedure has the advantage of simplicity, as it eliminates the necessity of a double centrifuging of each blood; it has the disadvantage of causing slight hemolysis.

The removal of native amboceptor undeniably results in an increased sensitivity; but it is a perhaps unnecessary complication if a flocculation test is carried out in parallel with the Wassermann, since the flocculation test ensures the detection of practically all serums which might be Wassermann negative because of their high content of native amboceptor.

Spinal fluid does not contain either native complement or amboceptor in significant concentration, and therefore requires neither inactivation nor the absorption of native amboceptor. The whole fresh fluid is used as such.

3. *Preparation of Complement.*—Fresh guinea pig serum, or serum kept overnight on the clot in the refrigerator, is diluted 1:10 by the addition of 9

volumes of salt solution. If the complement used in the test is a pooled preparation from 5 or more pigs, it is approximately uniform in activity, and the necessity for a complex and inadequate titration of hemolytic activity and "fixability" is eliminated. The complement can always be used in a fixed amount; and *the actual test mixture of antigen, serum and complement can be set up as the first step in the daily performance of the Wassermann reaction.* While the tests are in the ice box for their primary incubation (vide infra), one can then proceed to the preparation of the sensitized cell suspension.

The use of complement dried from the frozen state, and sealed in vacuo,[4] *introduces a marked simplification into the Wassermann technic.*[5] Because it is a pooled preparation, it is of uniform activity, and can be used in a fixed dilution; and since it retains its full hemolytic activity for ten months in the ice box, the time-consuming chore of frequent bleeding is eliminated.

4. *Setting Up the Tests.*—a. *Routine serum tests:* Many laboratories have found it more convenient to use 0.4 c.c. of each reagent instead of 0.2 c.c. as originally recommended. The set-up for the routine serum Wassermann is therefore as indicated in Table IV.

TABLE IV

THE ROUTINE SERUM WASSERMANN TEST

	SERUM CONTROL	TEST PROPER		ANTIGEN CONTROL	COMPLEMENT CONTROL (IN DUPLICATE)*			
Serum, c.c.	0.2	0.2	0.1	0.0	–	–	–	–
Complement, 1:10, c.c.	0.4	0.4	0.4	0.4	0.4	0.2	0.13	0.1
Antigen, 1:120, c.c.	–	0.4	0.4	0.4	–	–	–	–
0.85 per cent NaCl, c.c.	0.6	0.2†	0.3†	0.4	0.8	1.0	1.1	1.1

The tests are placed at 0° to 5° C. for 4 to 8 hours, followed by ½ hour at 37° C. Sensitized cells are then added (0.8 c.c. of a 1½ per cent suspension), and the results are read after 20 to 30 minutes at 37° C.

*One set is used to check the amboceptor titration; the second is incubated along with the tests as a check against complement deterioration (see text).

†May be omitted.

b. *The spinal fluid Wassermann, and the quantitative serum test:* For the quantitative titration of a known positive serum, the same test is carried out on a series of serum dilutions as outlined in Table V. The spinal fluid technic is

TABLE V

QUANTITATIVE WASSERMANN TEST FOR THE EXACT TITRATION OF A KNOWN POSITIVE SERUM

	SERUM CONTROLS		TEST PROPER					
Serum (undiluted), c.c.	0.2	0.05	0.2	0.05	–	–	–	–
Serum, 1:20, c.c.	–	–	–	–	0.4	0.2	0.1	0.05
Complement, 1:10, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Antigen, 1:120, c.c.	–	–	0.4	0.4	0.4	0.4	0.4	0.4
0.85 per cent NaCl, c.c.	0.6	0.75	0.2*	0.3*	–	0.2*	0.3*	0.35*

Complement and antigen controls are included as in Table IV. The tubes are incubated and the sensitized cells added as already described for the routine diagnostic tests (see Table IV and page 304). The six tubes of the test proper correspond to serum dilutions of 1:2, 1:8, 1:20, 1:40, 1:80, and 1:160; and the reagin content of any serum may be arbitrarily expressed as the maximum dilution yielding a positive result. Thus, a reading of + + + + + 0 corresponds to a reagin titer of 80, and a reading of + + + ± 0 0 corresponds to a titer of approximately 30, etc.

*May be omitted.

outlined in Table VI. The latter need be carried out routinely only down to 0.05 c.c.; but it is advisable to add the last three tubes when testing spinal fluids containing abnormally high quantities of protein, as such fluids may be strongly positive. The quantities of complement and antigen are halved in order to conserve spinal fluid.

TABLE VI

SPINAL FLUID WASSERMANN TECHNIC

	CONTROLS		TEST PROPER								
Spinal fluid,* c.c.	1.0	0.1	1.0	0.6	0.4	0.2	0.1	0.05	–	–	–
Spinal fluid, 1:10, c.c.	–	–	–	–	–	–	–	–	0.2	0.1	0.05
Complement, 1:10, c.c.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Antigen, 1:100, c.c.	–	–	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Four hours at 0° to 5° C., followed by ½ hour at 37° C. Add 0.4 c.c. sensitized cells. Read after twenty or thirty minutes at 37° C.

*Fresh whole fluid: inactivation of complement is unnecessary, and amboceptor need not be removed, since neither is present in spinal fluid in significant concentration.

The entire series of tests is now placed in the ice box for four to eight hours, followed by one-half hour at 37° C. *While the tests are in the ice box,* the sensitized cell suspension is prepared according to the following directions:

5. *Preparation of the Sensitized Sheep Red Blood Cell Suspension.*—a. *The preparation of the stock 3 per cent suspension:* Citrated sheep blood (five to ten volumes of blood into 1 volume of 5 per cent sodium citrate) or defibrinated blood is washed twice with ten volumes of salt solution. After the second washing, the cells are centrifuged in a graduated centrifuge tube until the volume of sedimented cells is constant. Ten to fifteen minutes at 1,500 to 2,000 revolutions per minute suffice. The measured volume of cell sediment is now resuspended in 32 volumes of salt solution to form the stock 3 per cent solution. Two cubic centimeters of blood usually yield 0.8 c.c. of sedimented cells, or 25 c.c. of the 3 per cent suspension, enough for 30 routine serum tests and the necessary controls.

b. *Titration of the rabbit amboceptor serum:* The technic of the amboceptor titration is given in Table VII. In the example there cited, a 1:3,000 dilution of amboceptor represents one amboceptor unit. The 3 per cent suspension of cells is now sensitized with an equal volume of amboceptor dilution containing 2½ units (in the example cited, a 1:1,200 dilution). One thus obtains a 1½ per cent suspension of cells sensitized with 2½ units of amboceptor.

c. *Check on the amboceptor titration: The amboceptor titration should now be checked by adding 0.8 c.c. of the sensitized cell suspension to one set of complement controls as indicated under* COMPLEMENT CONTROL *of Table IV.* This control consists of four tubes, containing 1, ½, ⅓, and ¼ the amount of complement used in the test. Since 2½ units of amboceptor automatically correspond to 2 to 2¼ units of complement, the tubes containing 0.4 and 0.2 c.c. of complement should hemolyze completely within thirty minutes, while that containing 0.13 c.c. of complement should show only partial hemolysis, and the last tube, which contains 0.1 c.c. of complement, should show little or no lysis. Any error in the amboceptor titration becomes immediately apparent, and can be readily rectified. The suspension is now ready for use and is placed in the ice box until needed.

6. *The Addition of the Sensitized Cells to the Tests and the Reading of the Results.*—After four to eight hours at 0° to 5° C., the tests are placed in a 37° water-bath for thirty minutes. After this secondary incubation, 0.8 c.c. of the sensitized cell suspension is added to all the tubes, which are then replaced at 37° C. The antigen control should be completely hemolyzed in ten minutes; and

TABLE VII

TECHNIC OF AMBOCEPTOR TITRATION

	AMBOCEPTOR DILUTION IN SALT SOLUTION*						
	1:1000	1:1500	1:2000	1:3000	1:4000	1:5000	0
Amboceptor dilution, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	-
3% cell suspension, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
0.85% NaCl, c.c.	0.8	0.8	0.8	0.8	0.8	0.8	1.2
Guinea pig complement, 1:10, c.c.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Example of reading of hemolysis after 30 minutes at 37° C.	Complete	Complete	Complete	Complete	Partial	Partial	None

A 1:3000 dilution therefore represents the minimal hemolytic concentration (one unit of amboceptor). The 3 per cent cell suspension is now sensitized by adding an equal volume of an amboceptor dilution containing 2½ units, e.g., a 1:1200 dilution in the example cited. The amboceptor titration is checked by adding the sensitized cell suspension to varying quantities of complement (see text, page 304). An amboceptor should not be used if it is so inactive that only the first two tubes of the amboceptor titration show complete hemolysis.

*These amboceptor dilutions are readily prepared as follows:						
1:1000 amboceptor, c.c.	0.4	0.27	0.2	0.13	0.1	0.08
Salt solution, c.c.	—	0.13	0.2	0.27	0.3	0.32
Final dilution of amboceptor	1:1000	1:1500	1:2000	1:3000	1:4000	1:5000

the complement control, incubated along with the tests, should show complete hemolysis in the first two tubes after twenty to thirty minutes (see preceding paragraph). The results of the tests are read after twenty to thirty minutes, whenever Tube 2 of the complement control, containing half the quantity used in the test, is completely hemolyzed.

If the serum control is not hemolyzed, the serum is *anticomplementary.* If the serum control is hemolyzed, and the other two tubes show no evidence of hemolysis, the result is *positive;* if either tube shows partial hemolysis, the result is *doubtful;* if both tubes are completely hemolyzed, the result is *negative.*

In the quantitative serum test, the result is expressed as the maximum dilution yielding a positive result. Thus a reading of $++++\pm 0$ corresponds to a "titer" of 40 to 80; and a reading of $+++000$ corresponds to a serum positive up to a 1:20 dilution, a "titer" of 20. Similarly, in the spinal fluid Wassermann, the result may be expressed as the minimum quantity yielding a positive result.

C. TECHNIC OF FLOCCULATION TEST

Minor modifications have been made in the original technic (1932). These are italicized in the following. The application of the flocculation reaction to spinal fluid is also described.

1. *The Serum Test.*—The clear antigen (containing 0.6 per cent each of cholesterol and corn germ sterol) is diluted by rapidly blowing 1.3 volumes of

4 per cent NaCl into 1 volume of antigen. If kept in the ice box, this dilution remains serviceable for *at least seven days.* Because its sensitivity gradually increases during the first forty-eight hours, the recommended procedure is to prepare the dilution at least one day in advance, allowing it to age in the ice box for twenty-four hours before use.

The serum to be tested is inactivated for *twenty* minutes at 56° C., and $\frac{1}{15}$ its volume of the antigen dilution added. Although the test can be carried out with 0.3 c.c. of serum and 0.02 c.c. of antigen dilution, it is more convenient to use twice these quantities. The turbid mixture is then shaken for two minutes. The incubation period can be adjusted at will to suit the circumstances.

If a rapid reading is necessary, as for an emergency transfusion, the tube is incubated at 56° C. for one-half hour. Otherwise, it is incubated at 37° C. for four to eight hours. After incubation, the tube is centrifuged at approximately 1,500 r.p.m. for ten to fifteen minutes. Three volumes of NaCl N/7 (0.85 per cent) are then added; i.e., three times the volume of serum used; and the results of the test are read.

In a negative reaction the tube is seen to be homogeneous and diffusely opalescent. On shaking, one sees a cloud of tiny refractile crystals, not visible if the tube is at rest. In a few serums, a little sediment may be deposited on the bottom of the tube, particularly if it was centrifuged at too high speed. This sediment, however, is not coherent and completely redisperses on mild agitation. In a *positive* reaction, the crystals clump to form coherent, coarse white flocculles floating in a clear and transparent fluid. There is a sharp contrast between the water-clear fluid and coarse flocculles of the positive test, and the homogeneous opalescence of the negative test.

One occasionally encounters weak positives, particularly in patients under antisyphilitic treatment. Usually, aggregation in such cases is definite; but even when there is only a slight granular appearance, not sufficiently marked to justify a definite reading of *positive,* a second centrifugation usually enables one to evaluate the results in terms of positive or negative. If aggregates are really present, they are thrown down in the salt: serum mixture to form a coherent flocculle at the bottom of the tube, covered by a clear supernatant fluid. In a negative result, the crystals remain discrete and are not thrown down: the tube remains homogeneous and opalescent. If the results cannot be read as definitely positive or definitely negative, the report is *doubtful.*

Although the macroscopic reading is more satisfactory, it is possible to read the results by microscopic examination. In a negative test one sees myriads of tiny crystals which do not cohere even though they are in immediate contact. In a positive test these crystals are clumped in much the same manner that red cells are clumped by an agglutinating serum, leaving clear spaces between the aggregates.

Reports are made as positive, doubtful, and negative.

2. *The Spinal Fluid Test.*—To 2 c.c. of *fresh* fluid is added 0.02 c.c. of the antigen suspension. It is to be noted that the fluid: antigen ratio (100:1) is much larger than the corresponding ratio in the case of serum (15:1). The tests are incubated for four hours at 37° C., centrifuged for ten minutes

(no salt solution is to be added), and the results read. In a *negative* result, the fluid is diffusely opalescent; in a *positive* result, the fluid is water-clear, and there is a tightly packed lipoid-reagin aggregate at the bottom of the tube. In a *doubtful* result, there is only partial aggregation of the crystalline particles of the antigen suspension.

D. SUMMARY

Modifications are described in the flocculation and Wassermann technics previously recommended. The most important of these are the use of absolute instead of 95 per cent alcohol in the preparation of the basic extract, and changes in the sensitization of this extract with sterols. For use in the flocculation reaction, the extract is fortified as heretofore with 0.6 per cent each of cholesterol and corn germ sterol. For the Wassermann reaction, however, the alcoholic extract is fortified with 1 per cent of cholesterol, and the corn germ sterol is omitted.

Minor improvements are pointed out in the technic of the Wassermann and flocculation reactions, which are described in detail.

The application of the flocculation test to spinal fluids is also described.

REFERENCES

1. Eagle, H.: Studies in the Serology of Syphilis. VIII. A New Flocculation Test for the Serum Diagnosis of Syphilis, J. Lab. & Clin. Med. **17**: 787, 1932.
2. Eagle, H.: Studies in the Serology of Syphilis. XIII. The Use of the Same Antigen for the Wassermann Reaction and the Author's Flocculation Test; and a Recommended Wassermann Technic, J. Lab. & Clin. Med. **19**: 621, 1934.
3. Eagle, H.: The Laboratory Diagnosis of Syphilis, St. Louis, 1936, The C. V. Mosby Co.
4. Flosdorf, E. W., and Mudd, S.: Procedure and Apparatus for the Preservation in "Lyophile" Form of Serum and Other Biological Substances, J. Immunol. **29**: 389, 1935.
5. Eagle, H., Strauss, H., and Steiner, R.: The Use in the Wassermann Reaction of a Uniform and Stable Dehydrated Complement, Am. J. Clin. Path. **5**: 173, 1934.

A UNIVERSAL ELECTRICAL DROP COUNTER OF SIMPLE DEVICE*

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GIBBS[1] in his review of drop recorders described an electrical recorder type in which the falling drops are utilized to close a contact between two platinum wires momentarily. This completes the circuit of a d.c. battery which operates an electromagnetic writer recording on a kymograph. This method is naturally restricted to liquids with a rather good electrical conductivity. Many body fluids, however, do not transmit enough current even if a considerable voltage is applied to the electrodes. Another disadvantage is the dependence of the electrode adjustment upon the size of the drops which, in turn, varies with the viscosity of the liquid, surface tension, and other factors. Hanike[2] therefore

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suggested a somewhat complicated arrangement, the idea of which was to make the recorded drops displace sodium sulphate in a vessel, thus forcing out a sodium sulphate drop which could be recorded electrically in the manner described above. This method is independent of the electrical conductivity of the fluid and is limited to measurements of the outflow only.

The arrangement suggested in this paper has the same purpose as Hanike's apparatus. It is based, however, on a different principle and is considerably

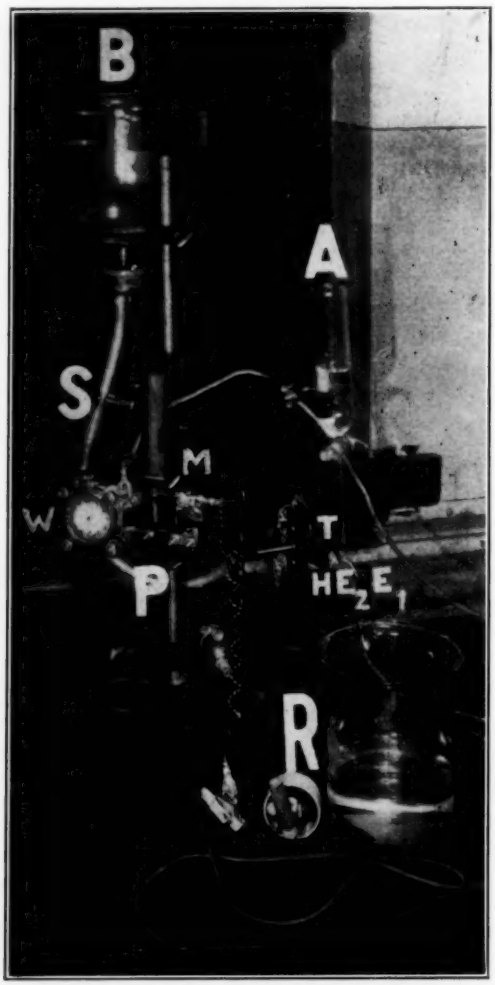

Fig. 1.—Drop counter set up. *B*, Reservoir for NaCl solution. *A*, Container for liquid the drops of which are to be counted. *P*, Bakelite plate. *W*, Counting watch. *M*, Magnet. *T*, Constricted glass tube. *H*, Electrode holder. E_1, Upper electrode. E_2, Lower electrode. *R*, Resistance.

simpler in construction. It may be used for counting drops of any liquid regardless of its electrical properties. The drops can either be recorded on a kymograph or their number read directly on an automatic counter. The whole equipment can be easily made out of a bell magnet and an Ingersoll watch, mounted

on a Bunsen stand (Fig. 1). A readjustment for different size of drops is scarcely required but, if necessary, can be accomplished by varying the height of the liquid container (A).

The characteristic feature of this device is the use of a liquid electrode. Two platinum wire electrodes, E_1 and E_2 (Fig. 2), are mounted together on a bakelite plate (P), E_2 being sealed in a glass tube (H) and E_1 being loosely fitted in another glass tube (T) which has a constriction at its right end.* A solution of sodium chloride of good electrical conductivity, from the reservoir (B) flows through the glass tube (T) and after passing through its constriction continues to flow along the electrode E_1, but still without making a contact be-

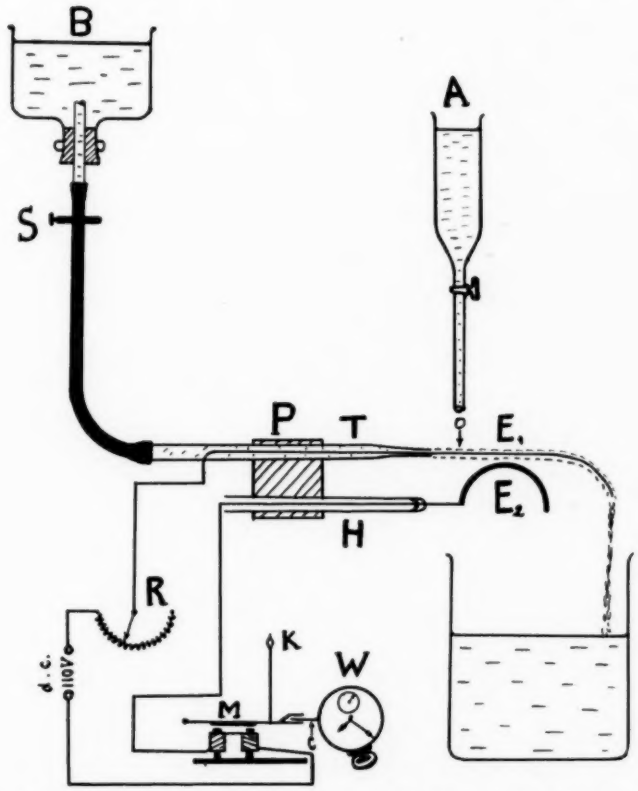

Fig. 2.—All letters correspond to Fig. 1. In addition: C, Copper wire attached to the escapement of the watch. K, Kymograph writer.

tween E_1 and E_2. One pole of a 110 V source (house voltage) is connected over a changeable (radio) resistance (R) to E_1 and the other pole over the magnet (M) to E_2. Now if a drop coming from A hits the saline beam in the neighborhood of the gap between E_1 and E_2 as indicated in Fig. 2, the beam will be deflected and will jump to E_2, thus forming a conducting bridge between the two electrodes and operating the magnet (M).† A writing lever (K) and a count-

*The free end of E_2 is formed into a semicircle, the apex of which is about 2 mm. below E_1. (The use of a thicker wire for E_2 is of advantage.)

†A magnet from an electrical bell is satisfactory.

ing watch (W*) are simultaneously operated by the magnet (M). This mechanism of making the contact can be easily demonstrated when the velocity of flow is reduced by regulation of the screw clamp (S). With a greater rate of flow of the salt solution this process becomes too fast to be observed. At the same time the duration of the contact becomes smaller which reduces the impulse communicated to the magnet (M). A slower rate of flow is therefore desirable, the limitation being that with too slow a flow the beam will not return back to E_1. The optimum velocity can be easily adjusted by regulating the screw clamp (S).

For slower counting rates a.c. may be used, but it is, however, by no means as reliable as d.c.

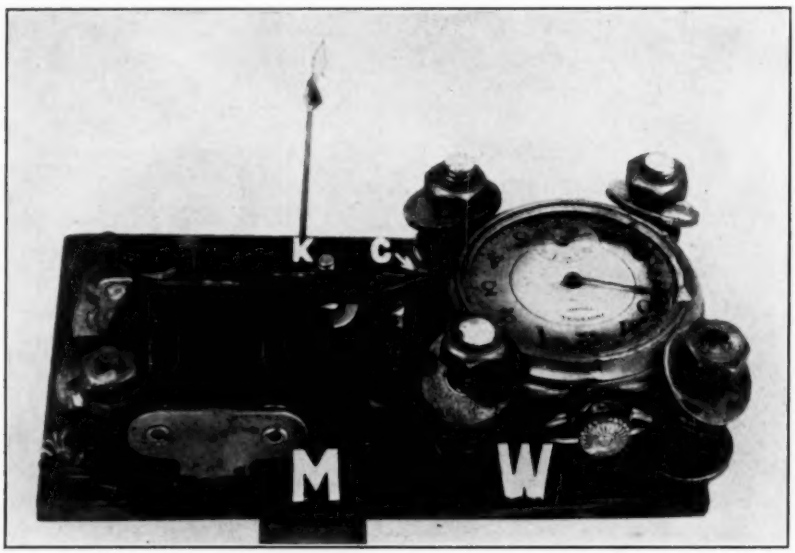

Fig. 3.—M, Magnet. K, Kymograph writer attached to the moving part of the magnet. C, Copper wire attached to the escapement of the watch. W, Ingersoll watch.

The counter operates best when E_1 lies above E_2, but it will still give satisfactory results if the plane of the electrodes is inclined as much as 45°.

Counting speeds up to 360 drops per minute can be recorded regardless of their size and composition.†

In order to secure stable conditions, the rate of flow has to be kept fairly constant. This can be obtained simply by making the difference in height between the reservoir B and the electrode E_1 much greater than the depth of B.

REFERENCES

1. Gibbs, O. S.: J. Lab. & Clin. Med. 12: 686, 1927.
2. Quoted by Gibbs: Ibid.

*The balance wheel of an Ingersoll watch was removed and a thin copper wire (C) leading out of the watch was soldered to the escapement. The motion of M can be easily used to move this wire so that for every drop the second hand will be moved by one-half second.

†This limit is mainly due to the inertia of the counting device. If only the kymograph recorder is used a much greater number of drops per minute can be recorded.

EXPERIMENTAL STUDIES OF THE SICKLING OF RED BLOOD CELLS*

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IT IS the purpose of this paper to report upon certain experiments which throw additional light upon the phenomenon of sickling in the disease called sickle-cell anemia, or drepanocytemia, and to describe a method which enables the observer to develop rapidly the sickling phenomenon in the blood of persons having the sickle-cell trait. The underlying mechanism responsible for the sickling phenomenon remains unknown. It is generally agreed that the sickling is a property of erythropoietic tissue and is not confined to the red cells of the circulating blood.[1-4] The trait is apparently inherent in the affected cells and is not due to any component of the plasma,[6, 7] though Josephs[8] reports that he has succeeded in removing the sickling-producing substance by washing the affected cells in salt solution.

Hahn and Gillespie[9, 10] in 1927 described a method for the production of sickle-cells in vitro from apparently normal but susceptible red blood corpuscles freely suspended in normal saline solution. The red cells were suspended in an oxygen-free atmosphere and were seen to assume the crescentic shape. As soon as oxygen was admitted to the chamber under a hanging drop, the sickled corpuscles immediately reverted to a circular form. It was found that the red corpuscles could be put through an endless number of transformations by admitting into the chamber alternate streams of CO_2 and O_2. Ethylene, nitrous oxide, nitrogen and hydrogen also induced the distortion of the red cells, provided the medium was slightly acid. Carbon monoxide acted just as effectively as oxygen in restoring sickle-cells to a circular form. These investigators advanced the hypothesis that the "formation of sickle-cells from susceptible corpuscles under a sealed cover slip is due to progressive decrease of oxygen and accumulation of carbon dioxide resulting from the metabolism of the blood cells." Diggs[11] was unable to confirm fully Hahn's experiments. We have been able to obtain excellent fixed preparations of sickled red cells for our course in Hematology, by making the smears from a proper specimen of oxalated blood in an atmosphere of carbon dioxide.

In the hope of throwing some light on the process of sickling, the influence exerted by certain dyes and other chemicals upon susceptible red blood cells has been studied.

Method and Material: The following "vital dyes" were used: *Neutral red* (15 drops 1 per cent alcoholic solution diluted to 10 c.c. with absolute alcohol), *brilliant cresyl blue* (1 per cent solution in 95 per cent alcohol), *Janus green* (1-2 drops 1 per cent alcoholic solution diluted to 10 c.c. with absolute alcohol), and *methylene blue* (2 drops 1 per cent alcoholic solution diluted to 10 c.c. with

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absolute alcohol). The solutions of these dyes were made up freshly in the concentrations usually employed for the "supravital" staining of living blood cells. Carefully cleansed glass slides were covered by the respective solutions and allowed to dry in the air. A drop of blood, obtained on a cover glass by finger puncture, was then placed on the prepared slides. The preparations were sealed with vaseline or paraffin. They were deemed satisfactory if a fairly uniform distribution of red blood cells was obtained without rouleau formation or marked overlapping of cells, if no air bubbles were present, and if the white blood cells took the dye properly and remained viable for at least four hours at 37° C. The quantitative sickling of red blood cells, if present, was then compared with that in simple, fresh blood preparations. In addition to the dyes mentioned above, sodium cyanide 1 per cent was used; white cell death was noted in these specimens in from fifteen to forty-five minutes.

Blood from 100 negro and 25 white patients was studied. These patients were in the hospital for various reasons and were chosen at random. All were adults ranging in age from twenty to fifty years. Since sickling was never observed in the blood of white patients, this group will not be discussed further.

The incidence of drepanocytemia and drepanocytic anemia in negroes has been estimated by several investigators, all of whom used moist unstained blood preparations, or fixed stained blood smears. Seydenstricker[12] found that 0.25 per cent of a group of Georgia negroes showed sickle-cell anemia, and that the frequency of true sickle-cell anemia, as compared with the incidence of the sickle-cell trait, was in the ratio of one to nine.[13] Myamoto and Korb[14] observed the sickle-cell trait in 6.3 per cent of a group of 300 southern negroes. Graham and McCarty[2] reported figures varying from 5.2 per cent to 7.2 per cent in a large group of St. Louis negroes. Lawrence,[15] working in Nashville, found that 5 per cent of the colored population showed the sickle-cell trait. Dolgopol and Stitt[16] report that the incidence of the sickling phenomenon was 6.5 per cent in the negroes at the Sea View Hospital, New York, and 5.2 per cent in a group of tuberculous negroes at Staten Island. Levy[17] found 5.8 per cent of sickling in the negroes at the Rockefeller Hospital. More recently Brandaw[18] reported that the sickle-cell trait occurred in 6.7 per cent of a group of 150 negro industrial workers in Houston.

From these reports one can say that the sickle-cell trait (drepanocytemia) may be expected to occur in about 6 per cent of a given negro population. In our group of negroes the sickle-cell trait was found in 7 per cent when moist unstained preparations were studied. In preparations stained supravitally, however, the incidence of sickling was found to be much higher, as shown in Table I.

TABLE I

PERCENTAGE OF SICKLING

METHOD	AFTER 12 HOURS	AFTER 24 HOURS	FINAL AVERAGE
Fresh, untreated preparations	5	9	7.0%
Neutral red preparations	5	10	7.5%
Janus green preparations	15	15	15.0%
Brilliant cresyl blue preparations	14	15	14.5%
Methylene blue preparations	14	14	14.0%
Sodium cyanide preparations	13	14	13.5%

It will be noted that the incidence of sickle red blood cells when stained supravitally with brilliant cresyl blue, janus green, and methylene blue, and in sodium cyanide preparations, was approximately twice that observed in untreated and neutral red preparations. The incidence of sickling in bloods treated with sodium cyanide was essentially the same as in those treated with janus green and brilliant cresyl blue.

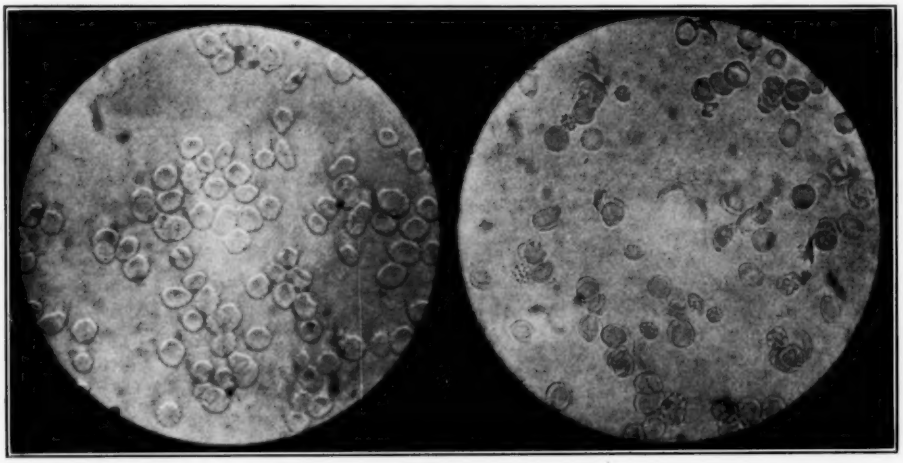

Fig. 1. Fig. 2.

Fig. 1.—Unstained moist preparation. Six hours old. Magnification ×1200.
Fig. 2.—Same preparation as shown in Fig. 1 at the end of twelve hours. Magnification ×1200.

Fig. 3.—Same preparation as shown in Fig. 2 at the end of twenty-four hours. Magnification ×1200.

Anemia, when present, did not materially affect either the degree or rate of sickling in the group of 100 patients studied, provided the red cell count was not below 2 million per mm. of blood. The highest incidence of sickling was observed in patients with only moderate anemia.

The high incidence of sickling observed in bloods stained supravitally and in bloods treated with sodium cyanide is very striking, being more than twice

that reported by other observers using a different technic. Frequently when no sickling could be demonstrated in fresh moist preparations, even when allowed to stand for twenty-four hours at 37° C., the same bloods showed sickling relatively early when stained supravitally.

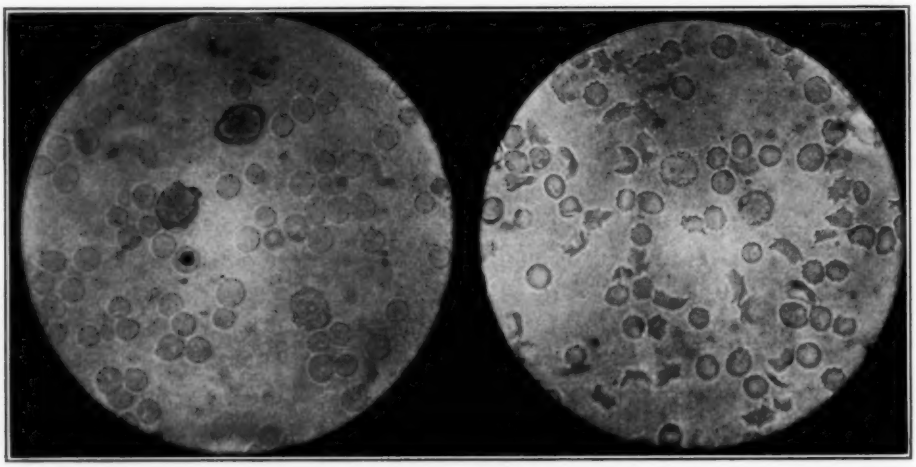

Fig. 4. Fig. 5.

Fig. 4.—Moist preparation stained supravitally with brillant cresyl blue, fifteen minutes old. Magnification ×1200.

Fig. 5.—Same preparation as shown in Fig. 4 at the end of two hours. Magnification ×1200.

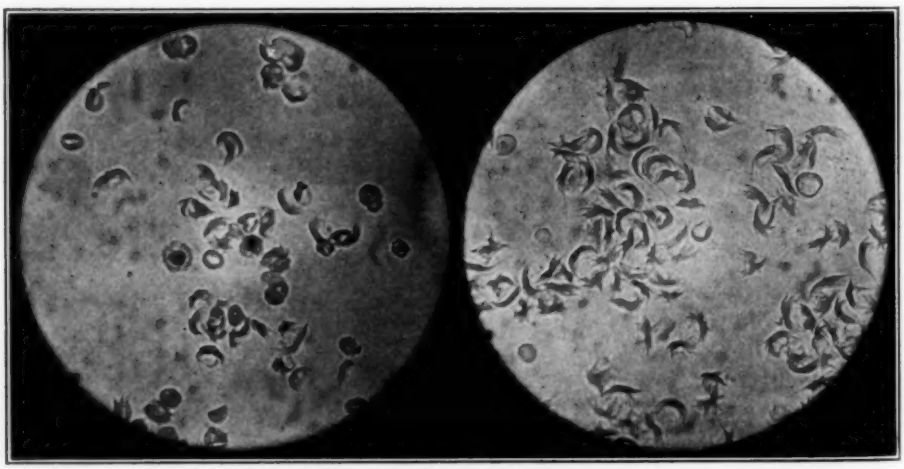

Fig. 6. Fig. 7.

Fig. 6.—Same preparation as shown in Fig. 5 at the end of three and one-half hours. Magnification ×1200.

Fig. 7.—Same preparation as shown in Fig. 6 at the end of five hours. Magnification ×1200.

Supravital staining with janus green, brilliant cresyl blue, and methylene blue, moreover, accelerates the rate of sickling remarkably. The rapidity of sickling, in patients showing the trait, when their bloods were studied in the usual moist preparations, was essentially as noted by Diggs[11] and others. When

these same bloods were treated by vitally staining dyes, however, or with sodium cyanide, the maximum degree of sickling was reached much sooner. The results are shown in Figs. 1 to 4.

DISCUSSION

Supravital staining by certain dyes accentuates the tendency and hastens the process of sickling in red cells possessing the sickling trait. Sickling thus produced is not an artefact as is clearly demonstrated by controls upon blood of both white and colored persons. It has been suggested that anoxemia plays an important rôle in activating the phenomenon of sickling. Since sodium cyanide, a reducing agent, acts upon susceptible red cells in apparently the same way as do the dyes used in the experiments, it seems probable that sickling is activated by the dyes by producing a condition of more or less profound anoxemia in the red cells.

SUMMARY AND CONCLUSIONS

1. By the simple technic of supravital staining with brilliant cresyl blue or janus green, the sickling trait is activated and accelerated. The maximum percentage of sickling is thus produced in from four to five hours, whereas the same result is only attained after twenty hours in unstained moist preparations.

2. With the use of the dyes mentioned, it was found that 14 per cent of an unselected group of 100 negroes showed the sickle-cell trait, as contrasted with reported average of about 6 per cent in groups of negroes where moist, unstained preparations were used.

REFERENCES

1. Seydenstricker, V. P., Mulherin, W. A., and Houseal, R. W.: Sickle Cell Anaemia. Report of 2 cases in children with necropsy, Am. J. Dis. Child. **25:** 132, 1923.
2. Graham, G. J., and McCarty, S. H.: Notes on Sickle Cell Anaemia, J. Lab. & Clin. Med. **12:** 536, 1927.
3. Levy, J.: Origin and Fate of Sickled Red Blood Cells, Arch. Path. **1:** 820, 1929.
4. Fradkin, W. Z., and Schwartz, L. S.: Sickle Cell Anaemia, J. Lab. & Clin. Med. **15:** 519, 1930.
5. Emmel, V. E.: A Study of the Erythrocytes in a Case of Severe Anaemia With Elongated and Sickle-Shaped Red Blood Cells, Arch. Int. Med. **20:** 586, 1917.
6. Huck, J. A.: Sickle Cell Anaemia, Bull. Johns Hopkins Hosp. **34:** 335, 1923.
7. Hein, G. E., McCalla, R. L., and Thorne, W.: Sickle Cell Anaemia, Am. J. M. Sc. **173:** 763, 1927.
8. Josephs, H. W.: Sickle Cell Anaemia, Bull. Johns Hopkins Hosp. **40:** 77, 1927. Idem: Clinical Aspects of Sickle Cell Anaemia, Ibid. **43:** 397, 1928.
9. Hahn, E. V., and Gillespie, E. B.: Sickle Cell Anaemia, Arch. Int. Med. **39:** 233, 1927.
10. Hahn, E. V.: Sickle-Cell (Drepanocytic) Anaemia, Am. J. M. Sc. **175:** 206, 1928.
11. Diggs, L. W.: The Rate of Sickling in Moist Preparations, J. Lab. & Clin. Med. **17:** 913, 1932.
12. Seydenstricker, V. P.: Sickle Cell Anaemia, South. Med. J. **17:** 177, 1924.
13. Seydenstricker, V. P.: Further Observations on Sickle Cell Anaemia, J. A. M. A. **83:** 12, 1924.
14. Myamoto, K., and Korb, J. H.: Menisocytosis (Latent Sickle Cell Anaemia), South. M. J. **20:** 912, 1927.
15. Lawrence, J. S.: Elliptical and Sickled Shaped Erythrocytes in the Circulating Blood of White Persons, J. Clin. Investigation **5:** 31, 1927.
16. Dolgopol, V. B., and Stitt, R.: Sickle Cell Phenomenon in Tuberculous Patients, Am. Rev. Tuberc. **19:** 455, 1929.
17. Levy, J.: Sicklemia, Ann. Int. Med. **3:** 47, 1929.
18. Brandaw, G. M.: Guidance of Sickle Cell Trait in Industrial Workers, Am. J. M. Sc. **180** (N.S.): 813, 1930.

Duke University

AN APPARATUS FOR THE AMPLIFICATION OF GLASS ELECTRODE POTENTIALS*

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THE glass electrode is theoretically so superior to all other apparatus for the determination of pH that it would seem logical for it to replace them completely. The slowness with which it has come to be a routine laboratory instrument is due to the difficulty of measuring potentials through its high resistance. More than 150 devices have been described in the literature, and this itself is an indication of the difficulty of the problem. Many of these devices are theoretically unsound or unworkable in practice. Recently, several commercial devices have been put on the market, which have sacrificed precision accuracy for portability and simplicity of operation. Glass electrode precision equipment, surpassing even the finest hydrogen electrode equipment in accuracy, is not yet available in commercially built apparatus.

The essential features of a satisfactory amplifier are sensitivity, a stable zero point and stability of the observed potential. The bases of the stability necessary for precision determinations are as follows:

The observation of variations in the plate current as an indication of the magnitude of the unknown potential applied to the control grid is subject to several sources of error. The plate current is not a linear function of the potential applied to the grid except over a limited range, and any calibration curve empirically obtained varies with the inevitable changes in the characteristics of the tube. Such devices depend on a continuous flow of current from the electrode system. This is another source of error, since the voltage drop across the glass membrane depends on its resistance with a consequent variation in the potential that is actually imparted to the control grid. Temperature changes produce enormous variations in the resistance of the glass, as has been shown by Morton (1934). Foshbinder and Schoonover (1930) have pointed out that the potential measured by such an apparatus is not the true value but is a value represented by the equation

$$E_d = E_t \pm i_g \cdot R_g$$

where E_d = observed potential, E_t = true potential, i_g = grid current, and R_g = cell resistance.

The advantage of such devices is that the plate current may be calibrated to read directly in terms of the pH of the unknown solution, but the instability of their calibration precludes their use for precise measurements.

Null-point instruments must be used if the highest accuracy is desired by a routine procedure. The unknown potential is opposed by the adjustment of a

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calibrated potentiometer until a galvanometer in the plate current indicates that the unknown system is not applying a potential to the control grid. Under such conditions no current is flowing from the electrodes and consequently variations in their resistance have no effect. The disadvantage of such an apparatus is that it requires a rather expensive potentiometer.

Consideration of the above equation shows that the operation of the tube must take place under conditions permitting the minimum flow of grid current. Even when a null-point instrument is at perfect balance, a very small current will flow from the grid, since the insulation is never infinitely great. This current disturbs the potential applied to the control grid because it is applied across the high resistance of the glass electrode. Nottingham (1930) gives a very full treatment of the disturbing effect of grid current. The devices of Stadie (1929), Partridge (1929), Elder (1929), Elder and Wright (1928), Schwarzenbach (1930), and many others are subject to error due to the flow of grid current. The grid current was minimized in the devices of Voegtlin, de Eds and Kahler (1930), Muller (1930), Foshbinder (1930), Du Bois (1930), and Partridge (1932).

Metcalf and Thompson (1930) have defined the causes of grid current. An understanding of the factors involved is essential in evaluating the many types of apparatus now available and therefore they will be briefly discussed.

1. *Leakage From the Grid Terminal Over the Surface of the Tube.*—This may be minimized by the use of tubes having the control grid contact at the top, and by keeping the glass surface clean and dry.

2. *Ionization of the Residual Gas in the Tube.*—Modern tubes are highly evacuated, although traces of gas are always present. Ionization can be prevented by using less than 8 volts on the plate. In actual practice it has been found possible to use 12 volts on the plate, provided the tube is especially "hard." The influence of this factor was reported by van der Bijl (1918).

3. *Emission of Electrons From the Grid Due to Heating.*—This is minimized by operating the filament at the lowest practical temperature. The disturbing effect of high temperature filaments has been reported by Kunz (1917), Pike (1919), and by Elder (1929).

4. *Emission of Positive Ions From the Filament.*—This is minimized by operating the filament at low temperature. This phenomenon has been studied by Wahlin (1929) and by Smith (1930).

5. *Photo-Electronic Emission From the Grid Caused by the Light From the Filament.*—This is minimized by operating the filament at low temperature.

6. *Photo-Electronic Emission From the Grid Caused by Soft X-rays Produced by the Anode Current.*—Such effects have been studied by Thomas (1925) and by Richardson (1926).

Perhaps one of the greatest sources of error in the operation of all types of glass electrode apparatus is insufficient insulation of the electromotively active surfaces of the electrode system. This electrical leakage shunts the glass electrode causing a lower potential to be observed. The magnitude of this error depends on the relative magnitudes of the resistance of the glass cell and of the resistance

to leakage. The equations of Morton (1934) show the relation between the true and the observed potential.

$$E = \frac{R + r}{r}$$

The per cent of error in the determination is

$$\frac{100\ (E-e)}{E} = \frac{100\ R}{(R + r)} = \text{per cent error}$$

where E = the true potential, e = observed potential, R = resistance of the glass electrode, and r = the parallel leakage resistance.

The elimination of this source of error depends on the proper mechanical construction of the apparatus.

Amplifiers should not depend on the use of sensitive galvanometers, since the difficulties attending their use nullify the advantage of an amplified system.

The batteries must be as few as possible and should be of high capacity. Variation in battery voltage is one of the prime sources of trouble. Some com-

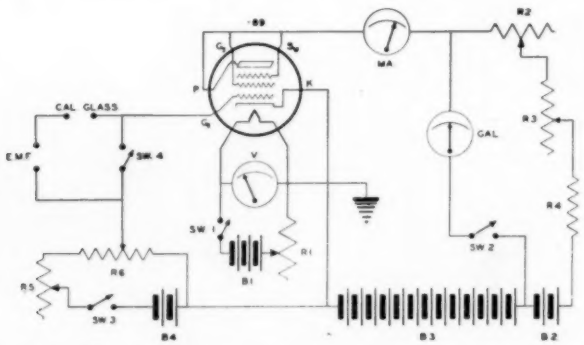

Fig. 1.—The plate (*P*), suppression grid (*Su*) and the screen grid (*Gs*) of the type 89 tube are connected. *MA*, 0-1 milliammeter; *V*, *0-10 DC.* voltmeter having a resistance of 1,000 ohms per volt; Gal, Leeds & Northrup No. 2320-D, having a sensitivity of 0.5 microampere per millimeter deflection, a coil resistance of 1,000 ohms and an external critical damping resistance of 2,400 ohms; *R1*, 20 ohms; *R2*, 400 ohms; *R3*, 10,000 ohms; *R4*, 1,500 ohms; *R5*, 400 ohms; *R6*, 20,000 ohms; *B1*, 6 volt storage battery; *B2*, 3 volts; *B3*, 195 volts; *B4*, 3 volts. The voltages of *B2*, *B3*, and *B4* are taken from the appropriate taps of a battery bank consisting of 5 radio "C" batteries each having a voltage of 4.5. *Sw.1*, *Sw.2*, and *Sw.3* may be conveniently controlled by a single 4 pole double throw Federal anticapacity switch. *Sw.4* is a single pole double throw Federal anticapacity switch. The *E.M.F.* leads attach to the measuring potentiometer.

mercial devices use small batteries, even for heating the filament, but this advantage of portability is obtained by a loss of stability.

The apparatus described below was designed to embody the greatest number of theoretical advantages with the simplest operation and construction consistent with precision accuracy. Measurements are easily and quickly obtained by unskilled operators with an error of ±0.008 pH unit. The apparatus may be briefly described as a null-point device operating at the free grid potential, with low plate voltage, and low filament temperature.

The tube operates as a triode. If Type 89 is used, the screen grid (Gs), the suppressor grid (Su), and the plate are connected. If Type 38 is used, the screen grid is connected to the plate circuit. This makes possible the relatively high amplification, even though the tube is operated much below its rated values.

The details of Fig. 1 are self-explanatory. All parts of the apparatus are enclosed in a grounded metal box except the galvanometer and the measuring potentiometer. The parts are mounted directly on the box with their controls external. A small jar of calcium chloride should be sealed inside the box to reduce surface leakages due to moisture. The procedure in operation is as follows:

1. Close switches *S1*, *S2* and *S3* and adjust *R1* so that the voltmeter (V) reads 2.5 to 3.5 volts. When tube 89 is used, reduce the filament voltage to the lowest value that will produce a plate current of 0.5 to 0.8 of a milliampere. Tube 38 should operate at about one-half of this plate current.

2. After a few seconds, when the ammeter (*A*) indicates that the plate current has reached its maximum, adjust *R2* and *R3* until the galvanometer reads approximately zero. The grid is now hanging free (at its free grid potential) and consequently the galvanometer is a little unstable, but this does not affect the accuracy of the instrument.

3. Close the tapping switch, *S4*, and adjust *R5* and *R6* until the galvanometer again reads approximately zero. The instrument is then balanced at the free grid potential of the tube. In this condition the galvanometer needle should be steady.

4. Release *S4*, and bridge the calomel cell to the unknown solution containing the glass electrode.

5. Adjust the measuring potentiometer until the galvanometer needle is not deflected when the tapping switch is closed. The needle may be poised at any readable part of the scale. It is only essential that it is not deflected by opening and closing the tapping switch.

The pH of the unknown solution is then calculated from the reading of the measuring potentiometer by the usual procedure.

REFERENCES

van der Bijl, H. J.: Theory of the Thermionic Amplifier, Physiol. Rev. **12**: 171, 1918.

Du Bois, Delafield: A Vacuum Tube Potentiometer Applicable for Use With Glass Electrodes of High Resistance, J. Biol. Chem. **88**: 729, 1930.

Elder, L. W.: pH Measurement With the Glass Electrode and Vacuum Tube Potentiometer, J. Am. Chem. Soc. **51**: 3266, 1929.

Elder, L. W., and Wright, W. H.: pH Measurement With the Glass Electrode and Vacuum Tube Potentiometer, Proc. Nat. Acad. Sc. **14**: 936, 1928.

Foshbinder, Russel J., and Schoonover, Janetta: An Improved Method of Measuring Glass Electrode Potentials, J. Biol. Chem. **88**: 605, 1930.

Kunz, J.: Amplification of the Photo-electric Current by the Audion, Phys. Rev. **10**: 205, 1917.

Metcalf, G. F., and Thompson, B. J.: A Low Grid Current Vacuum Tube, Phys. Rev. **36**: 1489, 1930.

Morton, Charles: The Effect of Electrical Leakage on the Electromotive Behavior of the Glass Electrode, J. Chem. Soc. **1934**: 256, 1934.

Muller, Friedrich: Theorie und Methodik der Elektronröhrenpotentiometer zur Messung electromotorischer Kräfte I, Ztschr. f. Electrochem. **36**: 923, 1930.

Nottingham, W. B.: Measurement of Small D. C. Potentials and Currents in High Resistance Circuits by Using Vacuum Tubes, J. Franklin Inst. **209**: 287, 1930.

Partridge, H. M.: A Vacuum Tube Potentiometer for Rapid E. M. F. Measurements, J. Am. Chem. Soc. **51**: 1, 1929.

Partridge, H. M.: A Zero Current Vacuum Tube Galvanometer, Mikrochemie **11**: 337, 1932.

Pike, Carl Eli: Amplification of the Photo-electronic Current by Means of the Audion, Phys. Rev. **13**: 102, 1919.

Richardson, O. W.: The Excitation of Soft X-rays, Proc. Roy. Soc. Lond. **A110**: 247, 1926.

Schwarzenbach, Gerold: Eine neue Vorrichtung zur Bestimmung von Potential en Zellen von sehr hoben inneren Widerstanden, Helv. Chem. Acta **13**: 865, 1930.
Smith, L. P.: The Emission of Positive Ions From Tungsten and Molybdenum, Phys. Rev. **35**: 381, 1930.
Stadie, William C.: An Electron Tube Potentiometer for the Determination of pH with the Glass Electrode, J. Biol. Chem. **83**: 477, 1929.
Thomas, C. H.: Soft X-rays From Iron, Phys. Rev. **25**: 322, 1925.
Voegtlin, C., De Eds, Floyd, and Kahler, H.: Electron Equilibria in Biological Systems. IV. An Adaptation of the Glass Electrode to the Continuous Measurement of Hydrogen Ion Concentration of the Circulating Blood, Public Health Rep. **45**: 2223, 1930.
Wahlin, H. B.: The Emission of Positive Ions From Metals, Phys. Rev. **34**: 164, 1929.

A SIMPLE METHOD FOR FIXING AND STAINING SPERMATOZOA*

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SINCE more and more emphasis is being placed on the morphology of spermatozoa in the appraisal of semen, it is essential that an undistorted picture of the individual spermatozoa be obtained, reasonably free of artefacts. This presents various problems, since the mucus and other elements of the semen make it nonadherent to the slide and cause a very disturbing sediment as a background which often distorts the picture. The spermatozoa do not readily stain with the more common stains sufficiently to distinguish the various parts, thus adding a further problem.

I have tried the various published technics with some success but have been dissatisfied with the results. Therefore, I have tried various combinations with a few modifications on my part and found that the following technic gave invariably good, clear, well-stained pictures.

Each step is important for good results. The specimen should be fresh, but it should be allowed to stand long enough for it to become liquid, since a high viscosity will prevent it from spreading in a thin even film over the slide.

1. Make a very thin smear on a slide in the same manner that a blood smear is made.
2. Shake rather vigorously until smear is dry.
3. Flame gently.
4. Flood slide with 0.5 per cent chlorozane for two minutes.
5. Wash gently with running water.
6. Flood slide with 95 per cent alcohol, let stand for one minute.
7. Tip off excess alcohol and allow to dry.
8. Stain with 0.5 per cent aqueous gentian violet three minutes.
9. Rinse with water.
10. Rinse with 95 per cent alcohol.
11. Rinse with water.
12. Counterstain with 1 per cent aqueous rose bengal one minute.
13. Wash with water.
14. Allow to dry.

REFERENCES

1. Meaker, S. R.: Human Sterility, Baltimore, 1934, The Williams and Wilkins Company, p. 109.
2. Williams, W. W., McGugan, A., and Carpenter, H. D.: The Staining and Morphology of the Human Spermatozoan, J. Urol. **32**: 201, 1934.

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DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

HEPATIC FUNCTION, Studies of, in Portal Cirrhosis and Congestive Heart Failure, Cantarow, A. Arch. Int. Med. **56**: 521, 1935.

Hyperbilirubinemia or abnormal retention of bromsulphalein or both were present in seventeen of twenty-two patients with portal cirrhosis. Visible jaundice was present in eight of twelve patients with hyperbilirubinemia.

Although ascites usually precedes the development of visible icterus, varying degrees of hyperbilirubinemia may be present in many cases before ascites can be demonstrated.

Retention of bromsulphalein was noted in 77.3 per cent of the cases in this series, being at times exhibited by patients with normal serum bilirubin values (seven cases). The relationship between retention of dye and the presence of ascites was not as consistent as was the relation of the latter to the degree of hyperbilirubinemia.

This dissociation of the retention of dye and that of bilirubin is practically never encountered in obstructive types of hyperbilirubinemia and suggests that primary impairment of the function of the hepatic cells is of fundamental importance in the production of hyperbilirubinemia in cases of portal cirrhosis. Similar observations were made in four cases of splenic anemia (Banti's disease).

Hyperbilirubinemia was present in ten of forty-two cases of congestive heart failure, in only two of which there was visible jaundice. Abnormal retention of bromsulphalein was present in fourteen cases. The severity of the condition appeared to be more consistently related to the degree of retention of dye than to the concentration of serum bilirubin.

Marked impairment of excretion of dye may occur in the absence of hyperbilirubinemia. Although not conclusive, this observation suggests that some factor, probably pulmonary infarction, other than hepatic functional impairment is necessary for the production of hyperbilirubinemia in patients with congestive heart failure.

BLOOD, Standards for Normal Children of School Age, Osgood, E. E., and Baker, R. L. Am. J. Dis. Child. **50**: 343, 1935.

Hematologic examinations are reported on the oxalated venous blood of 112 healthy boys and 103 healthy girls, aged from 4 to 13 years, inclusive.

The accurate methods used in making these examinations are briefly discussed.

There were no significant differences in these hematologic examinations with age or sex in this group.

The following values are recommended as normal for children of this age:

	Average	95 Per Cent Range
Erythrocyte count, millions	5	4.20 to 5.80
Hemoglobin, grams	12	10.00 to 14.00
Hemoglobin, coefficient	12	10.20 to 13.80
Cell volume, c.c.	36	31.00 to 41.00
Volume coefficient	36	31.00 to 41.00
Color index	1	0.85 to 1.15
Volume index	1	0.85 to 1.15
Saturation index	1	0.90 to 1.10

The results in these 215 children agree satisfactorily with those summarized from the literature.

The application of these normal standards to the diagnosis and treatment of anemia in children are discussed.

B. TUBERCULOSIS, Subsurface Growths in Solid Culture Media, Thuringer, J. M., and Butler, H. W. Am. Rev. Tubere. **31**: 466, 1935.

A subsurface growth of the Mycobacterium tuberculosis on various solid media has been shown to be a more or less constant phenomenon.

Further evidence is presented that this organism is capable of responding to its environment in many different ways (depending upon conditions of oxygen concentration, reaction of media, temperature, moisture, and its own metabolic products).

The authors are in agreement with those who believe that this organism occurs in various forms and that these pass through a cycle consisting of several phases, but we are unable to demonstrate the factors which bring about these changes.

It is of interest to note that most of the acid-fast rods were found in the subsurface growths, their profusion, arrangement, and acid-fastness depending upon the medium used. This may well lead to the question, "whether this subsurface growth does not resemble the actual status of this organism growing in the tissues more closely than the surface growth."

HEMORRHAGIC CONDITIONS, Effect of Moccasin Snake Venom in, Peck, S. M., and Rosenthal, N. J. A. M. A. **104**: 1066, 1935.

The use of snake venom as a means of controlling various types of hemorrhagic conditions has proved useful. It seems to be of value in most of the types of bleeding not associated with blood changes, such as epistaxis of long standing, uterine bleeding, hereditary multiple telangiectases (Osler's disease) and various forms of purpura, either toxic, allergic or endocrinal. In some instances, marked improvement of the secondary anemia was observed following the control of the bleeding. In some cases of achlorhydric anemia or anemia caused by long-standing uterine bleeding, iron was ineffective until snake venom was employed to control the bleeding.

Snake venom diminishes the bleeding tendency in certain cases of purpura hemorrhagica without affecting the blood picture. In some cases and occasionally in the chronic form, the blood picture returns to normal after the use of snake venom. This is not attributed to the effect of the venom but may be considered to be a spontaneous remission such as occurs without the use of this form of therapy. In such a case, however, the restoration of the blood picture to normal seems to be hastened by the reduction or control of the hemorrhagic condition.

Cases of hemophilia, thrombocythemia and leucemia show either no response or slight response at the beginning of treatment. Apparently snake venom has no appreciable effect on such conditions.

There are no contraindications to the use of snake venom. Allergic responses are rather mild. Exacerbations of the underlying conditions do not occur. Although the fundamental condition may not improve, the treatment does not exaggerate the hemorrhagic state.

Snake venom does not induce any changes in the blood picture. It does not affect the bleeding time, except in certain cases of thrombocytopenic purpura which respond to the treatment. In the allergic state resulting from the injection of snake venom an eosinophilia may supervene. This tends to subside after desensitization.

The administration of moccasin snake venom (Ancistrodon piscivorus) intracutaneously or subcutaneously (from 0.2 to 1 c.c. of a 1:3,000 solution) is effective in controlling various hemorrhagic conditions unassociated with blood changes. In thrombocytopenic purpura hemorrhagica, it seems to be effective in some cases and ineffective in others. It is of no value in cases of congenital hemophilia.

BLOOD, Hemoglobin Index and Jaundice of the New-Born, Hollosi, C., and Horvath, Z. Am. J. Dis. Child. **49**: 638, 1935.

The authors' studies lead them to conclude that:

The hemoglobin index of the newborn is less than 100 per cent when jaundice occurs, but more than 100 per cent if jaundice does not occur.

From the hemoglobin index one can foretell whether or not jaundice will occur.

Hemolysis does not necessarily precede jaundice. The formation of bilirubin can be attributed to some as yet unknown histologic function.

LYMPHOGRANULOMA INGUINALE, Value of Intradermal Injection of Serum as a Diagnostic Test for, Haynes, H. A. Arch. Dermat. & Syph. 32: 795, 1935.

Five patients with lymphogranuloma inguinale showing positive reactions to Frei antigen were tested under adequate conditions of control with intradermal injections of serum from patients known to have lymphogranuloma inguinale.

The mixture of Frei antigen and serum from a patient with lymphogranuloma inguinale produced the same cutaneous reaction as that to Frei antigen alone.

The intradermal injection of serum from patients with lymphogranuloma inguinale into patients showing positive reactions to Frei antigen caused no reaction, with one doubtful exception.

Therefore, it seems that the substitution of serum for Frei antigen is not satisfactory as a diagnostic procedure in cases of lymphogranuloma inguinale.

HEMATOPOIETIC EQUILIBRIUM, The, and Emergency Splenectomy, Doan, C. A., Curtis, G. M., and Wiseman, B. K. J. A. M. A. 105: 1567, 1935.

The pathologic physiology of the spleen may be manifest through either or both of two mechanisms (a) inhibitory, (b) destructive—and may affect any or all of the circulating blood elements.

The spleen is the major pathologic agent in congenital hemolytic jaundice.

Splenectomy is indicated as a prophylactic measure against clinical exacerbations of excessive hemolytic activity in the chronic and subacute manifestations of the disease.

Splenectomy is also the therapeutic procedure of choice in acute hemoclastic crises, whether the crisis is of spontaneous or of precipitated origin, and regardless of the severity of the anemia.

The immediacy of the erythrocyte response following splenectomy in hemolytic jaundice is dramatic, occurring on the operating table. It is usually a million or more cells per cubic millimeter in quantity and represents a true increase in total available circulating units. This autotransfusion removes the necessity for preoperative and/or postoperative transfusions.

Splenectomy is not contraindicated in properly selected cases of thrombopenic purpura in acute crisis, provided adequate preoperative blood transfusions are given. The immediacy of the beginning recovery and reappearance of blood platelets in the circulation following splenectomy in thrombopenic purpura may be quite as dramatic as the changes noted in hemolytic jaundice.

PLASMA CHOLESTEROL, Concentration in Glomerulonephritis and Other Terminal States, Cantarow, A., and McCool, S. G. Am. J. Clin. Path. 5: 516, 1935.

Determinations were made of the plasma cholesterol concentration in 18 patients (14 of whom died) with advanced chronic glomerulonephritis, 32 with nonnephritic nitrogen retention (26 of whom died) and 18 dying of conditions not accompanied by nitrogen retention. Although there was a distinct tendency toward fixation of the cholesterol concentration at a low level with increasing grades of nitrogen retention in both the nephritic and the nonnephritic groups, there was no constant quantitative relationship between the degree of cholesterolemia and of nitrogen retention. Low values were usually obtained in the group of terminal states not associated with nitrogen retention. No constant relationship was noted between the degree of hypocholesterolemia and of anemia.

The fact that similar findings are obtained in a variety of terminal states suggests that the development of hypocholesterolemia and its serious prognostic significance, under such circumstances, are probably related to the operation of some fundamental mechanism which is stimulated by a variety of pathologic states. Certain observations are reported which suggest that excessive withdrawal of cholesterol from the blood, as a result of abnormal stimulation of the activity of the reticulo-endothelial system, may be of importance in the pathogenesis of this phenomenon.

SPUTUM TYPING, Reliability of, and Its Relation to Serum Therapy, Bullowa, J. G. M. J. A. M. A. **105**: 1512, 1935.

The pneumococcus obtained from the sputum of patients ill with pneumonia is the type responsible for the disease in over 93 per cent of the cases in which confirmatory evidence was obtained by blood cultures, lung suctions, or metastatic foci.

The correct type is obtained from the first sputum in 71 per cent of the cases, from the peritoneum in 49.6 per cent of the cases, from the mouse's heart or brain in 21.4 per cent of the cases, and from the second sputum taken one or two days later in 5 per cent.

The Neufeld reaction gave the pneumococcus type in 76 per cent of cases.

The x group, or "type IV," is found to be responsible for only 1.2 per cent of cases.

The incidence of bacteriemia and the death rate are roughly parallel in most types of pneumococcic pneumonia.

Serum reduces the death rate in bacteremic and nonbacteremic cases and prevents bacteriemia in some types of pneumococcic pneumonia.

The action is specific and not a general protein therapy.

ANTICOAGULANTS, Magath, T. B., and Hurn, M. Am. J. Clin. Path. **5**: 548, 1935.

The standard deviation of the hematocrit test as performed with the Sanford-Magath tube is 0.60 per cent.

Heparin produces no swelling, crenation, or laking.

Dry oxalate produces a great deal of shrinkage of erythrocytes. When 22 mg. of dry oxalate per 10 c.c. of human blood is used, the average hematocrit value is 5.16 per cent less than that obtained with wet heparin, which indicates a shrinkage of 11.30 per cent. It is necessary to multiply the value for dry oxalate by 1.127 to equal the true hematocrit.

Sodium oxalate in 1.1 per cent solution gives an hematocrit value equal to that obtained with heparin and does not cause human erythrocytes to swell or become crenated nor does human blood become laked, as observed microscopically and spectroscopically, provided the centrifuging is done before two hours have elapsed. For practical purposes, this anticoagulant may be considered suitable for human blood. If the mixtures of oxalate and blood are allowed to stand the following factors must be used by which to multiply the per cent of erythrocytes: after two hours, 0.980; after four hours, 0.973; and after six hours, 0.969. If 1.6 per cent sodium oxalate be used, one must use the following factors: less than two hours, 1.030; two hours, 1.021; and four hours, 1.019; at six hours, the value is not significantly different from values obtained with heparin.

NEPHROSCLEROSIS, Malignant, MacMahon, H. E., and Pratt, J. H. Am. J. Med. Sc. **189**: 221, 1935.

Malignant nephrosclerosis from both a clinical and pathologic standpoint should not be looked upon as merely a progression of benign nephrosclerosis, but rather as a distinct and separate disease. It may occur alone or as a terminal complication of the benign disease. In the very early stages, when only the cardiovascular signs and symptoms are present, it may be impossible not only to say whether one is dealing with an early case of benign or malignant nephrosclerosis, but also it may be equally impossible to predict into which of these diseases the case will ultimately fall. As the disease progresses, the renal component becomes more and more conspicuous, and in the late stages it may be impossible to differentiate this disease from chronic glomerulonephritis. The etiology of benign and malignant nephrosclerosis has probably much in common, for one sees cases of chronic lead poisoning, pituitary basophilism, toxemias of pregnancy and so on, which on the one hand may show benign nephrosclerosis and on the other the much less frequent malignant disease. The course and prognosis depend not alone on the quality and quantity of the exciting agent but also in the manner in which the vessel wall responds. Where the response is of a simple degenerative nature, the disease progresses slowly, the prognosis is good and such cases are classed as benign nephrosclerosis. Where the vascular response is characterized by inflammatory changes of the intima, necrosis and hemorrhage, the course is more rapid, the prognosis is poor, and such cases are classed as malignant nephrosclerosis.

PREGNANCY, Investigation of New Biologic Test for Hormones in Pregnancy Urine. Kliener, I. S., Weisman, A. I., and Barowsky, H. J. A. M. A. **104**: 1308, 1935.

Group 1. Twenty-one female bitterlings were tested with known pregnancy urines. Of these, eight tests were negative, nine were definitely positive, and four showed some enlargement of the ovipositor.

Group 2. Urines from seven normally menstruating nonpregnant women were tested by the fish method, with the result that four gave positive ovipositor tests.

Group 3. Samples of urine from four different normal males were tested, with one positive result. This positive result was so striking and occurred so rapidly (before forty-eight hours) that the authors obtained another specimen from the same individual, a healthy normal man, aged 21. This was tested on six fish in separate aquariums with positive results in every fish within forty-eight hours.

Group 4. Three samples were obtained from women who were in the postclimacteric period and had not menstruated for from four to seven years. Each urine of this group was tested on two fish. One urine gave a positive fish test.

Group 5. The urines of Group 1 (from known pregnancies) were boiled and tested in the same way. In some cases two boiled urines were mixed and tested. The results were quite irregular, some boiled urines giving positive reactions and some negative.

It will be seen that only nine of the twenty-one urines from pregnant women gave definitely positive reactions. Of seven from normally menstruating nonpregnant women, four gave positive reactions. One male urine of the four tested was positive, and a later specimen from the same male gave positive reactions in every one of the six fish tested. Urines from women who had passed the menopause were positive in one of three cases. Boiled urines from pregnant women were positive in some instances and negative in others.

These results agree with those of Szusz and indicate that this biologic reaction is not a specific test for pregnancy as Kanter, Bauer, and Klawans intimate. Moreover, the latter authors do not report as great a variety of controls as we have and as Szusz previously reported. The author's finding that nonpregnant female urines, postmenopausal urines, male urines, boiled pregnancy urines, and physical disturbance may bring about lengthening of the ovipositor would seem to show that a standardization of a fish, as these authors suggest, would be extremely difficult if not impossible. Whether the phenomenon is due to the presence of estrogenic substance or to some other hormone or to some other substance remains to be determined.

TISSUE, Application of Blood Staining to Formalin-Fixed Tissues, Wilder, H. C. J. Techn. Meth. & Bull. Int. Assn. Med. Mus. **14**: 68, 1935.

1. Xylol.
2. Absolute methyl alcohol, 2 changes.
3. Cover slide with a freshly filtered saturated solution of Jenner's stain (National Aniline and Chemical Co.) in methyl alcohol, 3 minutes.
4. Add an equal amount of distilled water—1 minute.
5. Plunge, without washing, into a Coplin jar of dilute Giemsa solution (1 drop of Giemsa's spirochete stain from Hynson, Wescott and Dunning, to 1 c.c. of distilled water)—45 minutes.
6. Rinse and differentiate in acidulated distilled water (1 drop of glacial acetic acid to 5 c.c. of distilled water).
7. Rinse in pure distilled water.
8. Dehydrate quickly in 95 per cent alcohol and two changes of absolute alcohol.
9. Clear in xylol and mount in Canada balsam.

TISSUE, Rapid Staining Method for Spirocheta Pallida in Single Sections, Krajian, A. A. Arch. Dermat. & Syph. **32**: 764, 1935.

Drop fresh blocks of tissue about 5 mm. thick in a 10 per cent solution of formaldehyde heated to 67° C. (152.6° F.) and fix in an oven at 67° C. for ten minutes. (Tissues already fixed with formaldehyde do not require this treatment.)

Cut thin sections, from 5 to 10 microns, on a freezing microtome and place them in a 1 per cent aqueous solution of sodium cobalti-nitrite for five minutes.

Wash the sections in two changes of distilled water.

Place them in the following (No. 1) solution for fifteen minutes at 67° C.:

Uranium nitrate	1 gm.
Formic acid (chemically pure) 85 per cent	3 c.c.
Glycerin (chemically pure)	5 c.c.
Acetone	10 c.c.
Alcohol, 95 per cent	10 c.c.

Wash in two changes of distilled water.

Place the sections in a 0.75 per cent aqueous solution of silver nitrate for one hour at 67° C.

Rinse rapidly in distilled water.

Place the sections for three minutes in 2 c.c. of the developing solution, to which prior to use add 1 drop of egg albumen-glycerin fixative and thoroughly mix. (The egg albumen-glycerin fixative is that commonly used in fixing paraffin sections to slides.) While developing, work under a 60 watt electric lamp 4 feet above the specimen and expose the developing solution to light about fifteen minutes before it is to be used. The composition of the developing solution is as follows:

Hydroquinone	0.31 gm.
Sodium sulphite	0.006 gm.
Acetone	2.5 c.c.
Solution of formaldehyde (chemically pure) 40 per cent, made neutral	2.5 c.c.
Pyridine	2.5 c.c.
Saturated solution of mastic in 95 per cent alcohol	2.5 c.c.
Distilled water	15.0 c.c.

Wash for a few seconds in distilled water.

Place the sections in the 0.75 per cent solution of silver nitrate previously used from fifteen to twenty-five seconds.

Wash in two changes of distilled water.

Draw the sections onto slides and blot with fine filter paper; dehydrate in absolute alcohol for two minutes and then blot and clear in pure xylene for two minutes.

Mount the section in dammar resin.

COMMENT

The sections should be cut quite thin, preferably from 5 to 10 microns in thickness, as thicker sections make demonstration of spirochetes difficult owing to the fat content.

The purpose of using acetone and alcohol in the No. 1 solution is to remove any fatty substance present in the tissue, especially in sections of liver and brain.

The temperature of the oven is important; 67° C. has proved to be optimum. Lower temperatures require a longer time, while 70° C. or above has resulted in failure to demonstrate spirochetes in known control tissue.

Both the No. 1 solution and the solution of silver nitrate should be warmed to 67° C. before sections are placed in them. The No. 1 solution requires fifteen minutes and the solution of silver nitrate about thirty minutes to be heated to 67° C. Therefore, in order to save time it will be good practice to place the solutions in the oven before one begins to cut sections.

Control sections should always be prepared from material known to contain spirochetes.

The developing solution when made up fresh is a creamy color but soon turns brown. The solution keeps well from two to three weeks in a dark cool place. After that it begins to deteriorate, the mastic separating and settling to the bottom of the container. When this occurs, a fresh supply should be prepared.

The No. 1 solution is stable and keeps indefinitely.

Sodium cobalti-nitrite solution is not stable and should be made fresh each time.

All solutions and reagents should be kept in a dark cool place, preferably in a cabinet with black walls.

REVIEWS

Books and Monographs for Review should be sent direct to the Editor, Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

The Clinical Use of Digitalis*

DIGITALIS was an ancient remedy long before the publication of William Withering's epochal book, *An Account of The Foxglove and Some of Its Medicinal Uses.* That it is a valuable one is evidenced by the extensive literature which has accumulated concerning its characteristics and effects and the indications and contraindications to its therapeutic use.

The purpose of this book is to present clearly the various problems attendant upon the clinical use of digitalis and to survey the various data applicable to their solution.

The scope of the book is indicated by the Table of Contents: Effect on Ventricular Muscle, Effect on A-V Tissues, Effect on the Pacemaker of Normal Rhythm, Diuretic Effect, Effect on Blood Vessels and Blood Pressure, Electrocardiographic Effects, Toxic Effects, Indications, Use in Certain Special Situations, Dosage and Method of Administration, Contraindications and Dangers, and Digitalis and Prognosis.

The final chapter, "Therapeutic Theses," sums up concisely the conclusions regarding the clinical use of digitalis which at present appear to be indicated by the evidence presented in the preceding chapters. "If this has sometimes involved a measure of dogmatism unwarranted in reasoned discussion, the author trusts that these theses may yet commend themselves to thoughtful consideration and appropriate consideration."

These may be thus summarized: The therapeutic action of digitalis results from the action of the drug upon ventricular muscle, which muscular effect is of therapeutic value in "potential" heart failure. Digitalis lessens the energy requirement of the failing myocardium; it does not force it to work harder, but enables it to work better.

The indication for digitalis lies not in the manifestations of heart failure but in the heart failure itself.

Digitalis therapy must be considered primarily with reference to heart function, not to heart rhythm.

Diuresis from digitalis is a general circulatory effect; blood pressure changes are secondary circulatory effects. Pneumonia, shock, toxic states and septicemia are not indications for digitalis. Nor is angina pectoris.

While there are numerous other theses, based upon the data presented in this book, enough have been summarized to show that Dr. Luten has written an eminently practical book which should prove of great interest and still greater value to the physician.

Both author and publisher may be congratulated upon this book and, perhaps, most of all the physician who may find it in practical help in the solution of some harrassing clinical problem.

Diseases of the Nails†

IT IS, perhaps, a truism to say that the recognition of disease connotes the detection, recognition, and correlation of minutia; just as it may also be trite to recall that the majority

*The Clinical Use of Digitalis. By Drew Luten, M.D., Associate Professor of Clinical Medicine in the Washington University School of Medicine, and Physician to Barnes Hospital, St. Louis. Cloth, 226 pages, two plates, Charles C. Thomas, Springfield, Ill.

†Diseases of the Nails. By V. Pardo-Castello, M.D., formerly Assistant Professor of Dermatology and Syphilology, University of Havana, with a foreword by Howard Fox, M.D., Professor of Dermatology and Syphilology, New York University, Cloth, 177 pages, 94 illustrations. Charles C. Thomas, Springfield, Ill.

of patients seek the physician's advice because of signs and symptoms which in themselves may be of minor degree, though not always of minor significance.

In this book Dr. Pardo-Castello has excellently described disturbances of the nails, sometimes of importance as diseases of the nails per se, sometimes significant of underlying and more generalized disturbances, but always of importance to the patient.

Many of these without doubt are at times overlooked by the physician; many others, though seen, fail to receive due recognition of their importance or significance, largely because accurate information concerning them is likely to be the exception rather than the rule.

This may well be due to the fact that the nails and their disturbances have received heretofore relatively little mention in medical literature, a hiatus which this book completely fills.

Commencing, logically, with a discussion of the anatomy, physiology, and pathology of the nails, the text then discusses the affections peculiar to the nails, onychodystrophies, the ungueal manifestations of dermatoses and of systemic diseases, congenital affections of the nails, and the roentgen ray treatment of diseases of the nails.

An addendum lists the occupations in which diseases of the nails commonly occur and the ungueal symptoms due to poisons.

All in all this is a well-written, comprehensive, and excellently illustrated text which the physician may read with profit and which he will find most useful as a reference.

A Textbook of Pathology*

ONE of the finest textbooks of pathology comes out entirely reset in its sixth edition, with new material gleaned from the last four years, especially plentiful in the fields of vitamines, viruses, and endocrine functions. Dr. MacCallum's treatment of diseases as entities with their accompanying pathological alterations gives a fine concept of the diseases as pathological wholes in contrast with the treatment of pathological alterations as entities with accompanying lists of conditions in which they are found, preferred by some authors. This book will continue, by means of the new edition, to be an invaluable textbook for the student of both pathology and bacteriology.

Tissue Immunity†

DR. KAHN presents in this book original experimental studies dealing with the reactivity of the tissues themselves to the injection of foreign substances. Although it has long been known that an animal can be immunized by the injection of increasing amounts of organisms or their products and this immunity measured by humoral methods, Dr. Kahn has gone further and endeavors to tell us what tissue changes underlie the activities constituting this immunity. Likewise, desensitization of allergic persons has been practiced for some time, but it is the actual changes in the activity of the tissues producing the states of hypersensitivity and desensitization that have been studied here.

In each chapter there are experiments described with their results tabulated, and the theoretical considerations arrived at by the author from these results, which should be of great interest to the teacher, the research man, and the student of immunology. Following these are clinical considerations to aid the inquiring clinician in his understanding of the actual activities he is producing in the ever-increasing modes of prophylaxis, therapy, and diagnosis by parenteral methods.

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*A Textbook of Pathology. By W. G. MacCallum, Professor of Pathology and Bacteriology, The Johns Hopkins University, Baltimore. Sixth Edition. Entirely reset. 1277 pages with 697 illustrations, cloth. W. B. Saunders Company, Philadelphia and London, 1936.

†Tissue Immunity. By Reuben L. Kahn, M.S., D.Sc., University of Michigan, Ann Arbor. Cloth, 707 pages. Charles C. Thomas, Springfield, Ill., 1936.